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THE UNIVERSITY OF ALBERTA

MOLECULAR STUDIES OF THE NMEPHE PILIN OF *PSEUDOMONAS AERUGINOSA*

BY

BRITTAN L. PASLOSKE

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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3. Pasloske, B.L., Finlay, B.B. and Paranchych, W. (1985). Cloning and sequencing of the *Pseudomonas aeruginosa* PAK pilin gene. FEBS Letts. **183**, 408-412.
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Mark S. Peppler

Date: May 4, 1989

For my wife, Mary Léa,
who supports me in my personal growth,
for Maclean and Loic,
who teach me patience and unselfish love,
and for my parents, Al and Mary,
who have supported my education

ABSTRACT

The pili of *Pseudomonas aeruginosa* are extracellular organelles of 5.2 nm diameter and an average length of 2,500 nm. A pilus is composed of thousands of small identical protein subunits called pilin. These pilin are characterized by the unique N-terminal amino acid N-methylphenylalanine.

The pilin genes from different isolates of *P. aeruginosa* were cloned and sequenced. The DNA sequences revealed that the pilins are translated as a precursor form, with a positively charged, amino-terminal extension of 6 or 7 amino acids. A comparison of the translated pilin sequences established that there were four distinct linear domains consisting of a highly conserved, hydrophobic N-terminal region, and a central hypervariable domain flanked by two semivariable domains.

Pilin was detected mainly in its precursor form when it was expressed in *Escherichia coli*. It was localized primarily in the inner membrane. Provision of the pilin subunit alone did not lead to pilus assembly in *E. coli*.

When expressed in *P. aeruginosa*, deletions of 4 or 8 amino acids within the N-terminus of the pilin inhibited the removal of the leader peptide and the resulting pilins were unable to assemble into pili. A substitution of the fifth position glutamate by a lysine did not affect leader peptide removal but it did prevent the methylation of the N-terminal phenylalanine. These pilins assembled into heterologous pili in the presence of a wild-type subunit but they were unable to polymerize into a homogeneous structure.

Mapping surface residues on a pilus was acheived by selecting mutant pilins which lessened the binding of pilus specific bacteriophage to the assembled pilus. A glycine at position 113 substituted by a glutamate totally destroyed the binding of bacteriophage PO4. A serine at position 131 substituted by an asparagine prevented infection by a tip-binding phage. This serine is located within a C-terminal disulfide loop, suggesting that the disulfide loop is exposed at the pilus tip.

The cloned prototypes of five different pilin genes were provided to a pilin-minus mutant of *P. aeruginosa*. The susceptibility of each strain to four different pilus-specific bacteriophages was tested and a phage sensitivity profile was developed for *P. aeruginosa* expressing each pilin prototype.

A DNA sequence upstream of the pilin promoter was identified which positively regulated pilin gene expression. Deleting this region reduced pilin expression by 90%.

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Chapter I

INTRODUCTION

A. INTRODUCTION

Previous to the work presented in this thesis, the pili of *Pseudomonas aeruginosa* were known to be extracellular organelles with a diameter of 5.2 nm (Folkhard *et al.*, 1981) and an average length of 2,500 nm (Bradley, 1972). These pili acted as the receptors for a number of pilus-specific bacteriophages (Bradley and Pitt, 1974). The protein monomer, pilin, had an approximate molecular weight of 15,000. Thousands of pilins were organized in a helical array of 5 subunits per turn to form the pilus structure (Fig. 1). *P. aeruginosa* is an opportunistic pathogen causing local infections at burn sites or in damaged corneas as well as systemic infections in cancer patients or burn victims (Bodey *et al.*, 1983). It is also the major pathogen of nosocomial pneumoniae and chronic lung infections in patients with cystic fibrosis (Kohler and White, 1979). The pili were shown to be a virulence factor due to their ability to mediate adherence of *P. aeruginosa* to epithelial tissues, thus promoting colonization and infection (Doig *et al.*, 1988).

Protein sequencing was used to determine the complete sequence of the pilin from one isolate of *P. aeruginosa*. The N-terminus had a unique N-methylphenylalanine (NMePhe) and hence, these pilins belonged to the NMePhe class of pili (Paranchych and Frost, 1988).

The NMePhe was followed by a hydrophobic stretch of 28 amino acids which were thought to be involved in subunit-subunit interaction in the assembled pilus. At the C-terminus were two cysteines in the oxidized state, resulting in a disulfide loop of 12 amino acids (Watts *et al.*, 1983b). Preliminary studies indicated that this loop was involved in the binding of the pilus to epithelial tissue (Paranchych *et al.*, 1986).

In this thesis, there are four main areas addressed concerning the pili of *P. aeruginosa* at the molecular level: 1) the cloning and sequencing of the pilin genes from different isolates of *P. aeruginosa*, 2) the expression, maturation, and assembly of wild-type and mutant pilins in *Escherichia coli* and *P. aeruginosa*, 3) the mapping of surface residues on the pilus, and 4) the regulation of pilin gene expression. The following is a brief summary of the pertinant findings in these studies.

DNA sequencing of pilin genes cloned from different isolates of *P. aeruginosa* revealed that not many more than five pilin prototypes probably exist. A comparison of the prototype pilin sequences led to the observation that the pilin can be divided into four linear domains. One domain was composed of the first 28 amino-terminal amino acids which are strongly hydrophobic and highly conserved among the five pilins. In contrast, a central, hypervariable region (the immunodominant epitope) which is flanked on either side by two semivariable domains. It was found that pilin is translated in a precursor form, with a positively charged, 6 or 7 amino acid amino-terminal extension which is removed before pilus assembly.

The expression of the pilin protein in *E. coli* was found to be weak unless a strong promoter is provided upstream of the pilin gene. Pilin translated in *E. coli* is localized primarily in the cytoplasmic membrane. Much of the pilin was detected as the precursor form, suggesting that *E. coli* does not have the enzymes to efficiently remove the short leader peptide. Even though pilin is expressed and transported into the membrane, the subunits did not assemble into pili in *E. coli*. The inefficient processing of prepilin and the abscence of polymerized pilin demonstrates that *E. coli* probably lacks many of the accessory genes required for pilus assembly.

A truncated pilin consisting of the first 36 amino acids of prepilin (the leader peptide plus the hydrophobic N-terminal region) was able to associate with the membrane fraction of *E. coli* indicating that this region is important in the translocation of pilin into the membrane.

Maturation of the nascently, translated *P. aeruginosa* pilin was found to be a two step process. The short leader peptide was first enzymatically removed and this was then followed by the methylation of the new N-terminal amino acid, phenylalanine. Mutations within the mature portion of the N-terminus of the pilin was able to affect either of these processes. In studies of pilin expression in *P. aeruginosa*, the deletion of either 4 or 8 amino acids in the N-terminus prevented the cleavage of the leader peptide. Although these pilins were able to translocate into the inner membrane, they were unable to assemble into pili. If a glutamate at position 5 was substituted by a lysine, the leader peptide was efficiently removed but methylation of the N-terminal phenylalanine was inhibited. These mutant pilins did not assemble as a homogeneous structure but in the presence of wild-type subunits they assembled as heterologous pili. The polymorphic pili had a wavy-braided appearance, unlike the straight filaments of the wild-type pili.

The clones of each of the five prototype pilin genes were expressed and assembled in a single heterologous strain of *P. aeruginosa*. These findings indicated the broad capacity that the pilus machinery has for different pilin alleles. Phage-susceptibility profiles were developed for *P. aeruginosa* expressing each of the five pili prototypes, using four different pilus-specific bacteriophage. It was concluded from this study that each of the four phage recognized a different receptor-binding domain on the pilus.

Using similar techniques, single point mutations within the pilin subunit were selected which abolished the binding of pilus-specific bacteriophage, suggesting that these residues were exposed on the surface of the pilus. A glycine at position 113 substituted by glutamate obliterated the adherence of bacteriophage PO4. The substitution of a serine within the disulfide loop at the C-terminus prevented infection by a tip-binding phage. This finding is significant because it indicates that the disulfide loop, which is probably the receptor-binding domain for an epithelial cell pilus receptor, is exposed at the pilus tip.

Pilin gene expression was found to be positively regulated by a DNA sequence upstream of the pilin promoter. Deleting this sequence reduced pilin gene expression by more than 90%. The pilin gene has a promoter which resembles an NtrA-dependent promoter. Thus far, genes requiring the NtrA sigma factor for expression also require another protein factor for maximum expression.

The sections below provide additional detail regarding the development of experimental strategies as well as background literature pertaining to analogous systems in the other bacteria which produce NMePhe pili.

B. CLASSIFICATION

P. aeruginosa belongs to a group of many other bacteria which produce a unique form of translocation called twitching motility (Henrichsen, 1983). The cells move predominantly singly and the motion is intermittant and jerky. This movement leads to the production of spreading zones on solid surfaces. Members of this group include species from such diverse genera as *Acinetobacter*, *Neisseria*, *Eikenella*, *Moraxella*, *Bacteroides* and many different species of *Pseudomonas*. Pili extending from the poles of these bacteria are responsible for twitching. These polar pili belong to the N-methylphenylalanine (NMePhe) class of fimbriae. The subunit monomer of pili, pilin, has the unique N-terminal amino acid NMePhe as determined by protein sequencing in *Bacteroides nodosus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella bovis* and *P. aeruginosa* (McKern *et al.*, 1983; Hermodsen *et al.*, 1978; Marrs *et al.*, 1985; Frost *et al.*, 1978). Protein sequencing demonstrated a highly conserved, hydrophobic N-terminal region of approximately 30 residues among the NMePhe pilins from these different species and from the pilin of *Moraxella nonliquefaciens* (Froholm and Sletten, 1977).

C. ANTIGENIC VARIATION OF PILIN

Pilins from *N. gonorrhoeae*, *B. nodosus* and *P. aeruginosa* have been sequenced entirely by protein chemistry (Sastry *et al.*, 1983; McKern *et al.*, 1983; Schoolnik *et al.*, 1984). The pilins had molecular weights ranging from 15,000 to 18,000. Comparison of the protein sequences indicated little homology among these pilins except at the N terminus. Weak homologies among the pilins were understandable considering that they were isolated from different genera. However, even more interestingly, serologically different NMePhe pili were identified among isolates within the same species. For instance, Bradley and Pitt (1975) serotyped the NMePhe pili of six different strains of *P. aeruginosa* and learned that only two of the strains had pili which were serologically alike. In *B. nodosus*, eight different serogroups for the NMePhe pili have been identified (Claxton *et al.*, 1983) while in *M. bovis*, a single strain is capable of alternately producing two types of pilins of molecular weights 16,000 and 18,000 (Marrs *et al.*, 1985). Early studies of the pili of *N. gonorrhoeae* indicated that pilin undergoes extensive antigenic variation. Buchanan (1975) found that the pili from many different clinical isolates were immunologically distinct.

With the advent of molecular biology, it became possible to begin exploring the genetic mechanisms responsible for creating this antigenic diversity. *B. nodosus* has a single copy of the pilin gene per genome (Elleman, 1988). The various pilin serotypes have seemingly arisen via antigenic drift. On the basis of nucleotide sequences of pilin genes from many different isolates, *P. aeruginosa* appears to be similar to *B. nodosus* in this regard. In contrast, the alternate expression of either the alpha or beta pilin subunits in *M. bovis* strain EPP63 is due to the inversion of a 2-kilobase region of DNA (Marrs *et al.*, 1988). Even more complicated is the mechanism of pilin variation in *N. gonorrhoeae*. Studies have shown that a single cell can give rise to many different progeny which express antigenically distinct pili (Hagblom *et al.*, 1985). In strain MS11, there is one locus (*pilE1*) responsible for the expression of an intact pilin. Another locus, *pilS1*, is a region of 3 kilobases which contains six variant pilin sequences. These pilin gene variants in the silent locus are capable of recombining with the pilin gene in the *pilE1* expression site, thus establishing new, distinct pilin genes in the expression site. Interestingly, the recombination event is intergenomic and not intragenomic (Seifert *et al.*, 1988). Piliated gonococci are highly competent for DNA transformation and therefore, suitable for the uptake of DNA released from other autolysed gonococci.

D. PILIN EXPRESSION, TRANSLOCATION AND PROCESSING

Besides being concentrated into extracellular appendages, the pilin of *P. aeruginosa* is localized in pools in the cytoplasmic and outer membranes (Watts *et al.*, 1982). Most proteins of gram-negative bacteria secreted beyond the barrier of the cytoplasmic membrane are translated as larger, precursor forms (Michaelis and Beckwith, 1982). They have an extended amino terminus of 15 to 25 amino acids which is enzymatically removed as the protein enters the inner membrane. DNA sequencing of the NMePhe pilin genes revealed that the leader peptide is unusually short, constituted by only 6 to 7 amino acids. This novel aspect of *P. aeruginosa* pilin made it an interesting candidate for secretory studies. Initial protein expression studies are usually performed in *E. coli* because most of the methodologies and expression systems have been developed for *E. coli*. Therefore, the first protein secretion experiments in this study were designed to assay for the expression of a cloned *P. aeruginosa* pilin in *E. coli*. Further experimentation was aimed at determining whether *E. coli* had the enzymes to properly remove the novel leader peptide and then to methylate the newly exposed phenylalanine. It was also important to ascertain whether the provision of the pilin gene alone would lead to pilus assembly. The likelihood that pilin self-assembly would occur was poor since at least 11 gene products, besides pilin, are required for the formation of F-pili in *E. coli* (Ippen-Ihler and Minkley Jr., 1986). As well, the operon of type 1 pili in *E. coli* has a total of 9 genes (Klemm and Christiansen, 1987).

The expression of several different NMePhe pilins in *E. coli* has been studied. *M. bovis* and *B. nodosus* pilin genes transformed into *E. coli* weakly express a pilin product which localizes primarily to the inner membrane fraction (Marrs *et al.*, 1985; Anderson *et al.*, 1984). The *B. nodosus* pilin do not polymerize into pili in *E. coli.* Almost identical results were obtained for the expression of *P. aeruginosa* pilin in *E. coli*.

E. STRUCTURE/FUNCTION STUDIES

There is a paucity of literature in the field of NMePhe prepilin translocation, maturation, and assembly. To delineate important regions of pilin for these processes, the pilin gene was subjected to several forms of tampering: deletion mutagenesis, chemical mutagenesis, transposon mutagenesis and site-specific mutagenesis. These mutations were localized to the N-terminal region of the pilin. Beyond assessing the effects of the mutations regarding pilin maturation in *E. coli* and *P. aeruginosa*, these pilin mutants provided information related to pilus assembly in *P. aeruginosa*. These experiments identified amino acid residues required for leader peptide removal, the methylation of the N-terminal phenylalanine and for pilus assembly.

The development of isogenic, pilin-lacking strains of *P. aeruginosa* by gene replacement methodology was of major importance for these and other experiments. A host had been engineered in which an exogenously supplied pilin gene (via a plasmid) could be studied without background interference from the chromosomally encoded pilin gene. The potential applications of this pilin-lacking mutant are numerous, including the ability to easily discern the assembly of heterologous or mutant pilin subunits by electron microscopy. Previous to the construction of the pilin-lacking mutants, it was difficult distinguish the pili assembled from an endogenous pilin gene compared to the pili polymerized from plasmid encoded gene products.

F. HETEROLOGOUS PILI EXPRESSION

The conserved N-terminal region of NMePhe pilin would lead one to predict that the pilin gene from one genus will be expressed and assembled into pili in an NMePhe host from a different genus. Indeed, both Elleman *et al.* (1986) and Mattick *et al.* (1987) produced *B. nodosus* pili in *P. aeruginosa* by providing just the *B. nodosus* pilin gene. These experiments demonstrated the breadth the pilus machinery has for heterologous pilins and indicates that one strain of *P. aeruginosa* is capable of assembling pilin from a number of other heterologous strains of *P. aeruginosa*.

To demonstrate that *P. aeruginosa* is able to assemble heterologous *P. aeruginosa* pilins, the pilin genes from five different *P. aeruginosa* isolates were cloned into a broad host range vector. They were introduced into the pilin-lacking *P.*

aeruginosa strains and assayed for the assembly of pili. The results were consistent with those obtained from the *B. nodosus* pili expression experiments in that all the pilin protoypes were assembled (Elleman *et al.*, 1986; Mattick *et al.*, 1987).

G. THE DISULFIDE LOOP

All the NMePhe pilin genes sequenced have at least two cysteine residues which are usually located at the C terminus of the protein. The spacing between these residues varies from 12 to 17 amino acids for *P. aeruginosa* and *B. nodosus* (serogroups D and H) to about 30 amino acids for gonococcal pilin (Sastry *et al.*, 1983; Elleman, 1988; Schoolnik *et al.*, 1984). The *B. nodosus* pilins (serogroups A, B, C, E, F, and G) have centrally located cysteines with approximately 40 amino acids between the cysteine residues (Elleman, 1988). Studies on several different NMePhe pilins determined that these cysteines are in their oxidized state, thus resulting in the formation of a disulfide loop (Schoolnik *et al.*, 1984; Watts *et al.*, 1983b).

To date, work involving the disulfide loop has been limited to the pilins of *N. gonorrhoeae* and *P. aeruginosa*. With respect to *N. gonorrhoeae*, Nicolson *et al.* (1987) demonstrated that type-specific monoclonal anti-pili serum directed to the disulfide loop were able to inhibit adhesion of purified pili to human buccal epithelial cells and they were able to protect Chang epithelial cells against homologous gonococci in a cytotoxic assay.

In early studies, Paranchych *et al.* (1986) found that a proteolytically derived peptide from *P. aeruginosa* pilin, encompassing the disulfide loop, was able to inhibit the adherence of the homologous bacteria. More recently, antipeptide antibodies to a 17-residue oxidized peptide, containing the sequence of the disulfide loop, was able to cross-react with both homologous and heterologous pili (Lee *et al.*, 1989). As well, a monoclonal antibody to the disulfide loop of strain PAK pilin was able to prevent adherence of the homologous strain to buccal epithelial cells (P. Doig, P.A. Sastry, R.S. Hodges, K.K. Lee, W. Paranchych, and R.T. Irvin, submitted). The above observations provide strong evidence that the disulfide loop is exposed at the pilus surface acting as the receptor-binding domain which recognizes epithelial cell receptors. These results, in conjunction with the data provided in this thesis, strongly suggests that that the disulfide loop is exposed at the pilus tip and therefore, the receptor-binding domain is located there also.

H. REGULATION

From S1 nuclease mapping of the pilin mRNA transcript in *P. aeruginosa*, Johnson *et al.* (1986) recognized a sequence just upstream of the transcription start point similar to an NtrA-dependent promoter. NtrA, the gene product of *ntrA*, is a sigma factor initially discovered to be involved in the transcription of genes which fix and assimilate nitrogen in organisms like *Klebsiella pneumoniae* and *E. coli* (Gussin *et al.*, 1986). Other genes of *Pseudomonas* have NtrA-like promoters such as the *xylABC* operon of the TOL plasmid (Dixon, 1986) and the carboxypeptidase G2 gene (Minton and Clarke, 1985). Most of the NtrA-dependent promoter systems examined have proteins which act as positive regulators. NifA, NtrC and XylR enhance the transcription of the nitrogen fixation genes, the nitrogen assimilation genes, and the *xylABC* operon, respectively. Specific consensus NtrC- and NifA-binding sequences are localized upstream of the NtrA-dependent promoter. In light of this information, the DNA sequence upstream of the *P. aeruginosa* NtrA-like pilin promoter was searched for sequences homologous to the NifA- and NtrC-binding sequences. A sequence resembling a NifA-recognition site was identified and deletion mutagenesis was used to establish that an upstream sequence contributes to the regulation of pilin gene transcription.

Vibrio cholera pilin is very much like NMePhe pili in the first 25 amino acids except that the mature N-terminal amino acid is a methionine instead of an NMePhe. The pilin gene (*tcpA*) of *V. cholera* is positively regulated by ToxR, a protein which also modulates the expression of the cholera toxin genes. In fact, it appears that ToxR coordinately regulates the expression of at least 17 gene products in *V. cholera*, all of which are secreted proteins (Peterson and Mekalanos, 1988). Interestingly, ToxR is a transmembrane protein of the inner membrane responding to multiple physiological parameters such as pH, salt, and nitrogen levels. It is postulated that the periplasmic domain is able to sense environmental changes and these changes are transduced to the cytoplasmic domain which activates gene transcription in an unknown manner (Miller *et al.*, 1987). ToxR also falls into a class with many other transcriptional activators. Its N-terminal region has homology with the C termini of OmpR, PhoM, PhoB and other activators (Miller *et al.*, 1987).

The pilin gene of *N. gonorrhoeae* has both a positive (*pilA*) and a negative (*pilB*) regulator of transcription, both of which act in *trans*. These genes are expressed in the opposite orientation of each other and the 5' ends of the two genes overlap. The two loci map just over 3 kilobases downstream of the *pilE* expression locus. Neither of these two regulators have immediate apparent homology to ToxR or the other transcriptional regulators mentioned above.

Hobbs *et al.* (1988) documented that there is a positive regulator for the pilin gene of *P. aeruginosa*. A 1.2-kilobase fragment containing the *P. aeruginosa* pilin gene, when transformed into *Pseudomonas putida*, produced 30-fold lower levels of pilin mRNA than did a 6.2-kilobase fragment containing the pilin gene centered within it. More work will be needed to localize the gene responsible for the enhanced transcription. It will be interesting to learn whether it has homology with ToxR or *pilA* of *N. gonorrhoeae*.

I. CONCLUSIONS

Although there are similarities among the different NMePhe organisms regarding the homologies of the N-termini of the pilin subunits and the expression of these monomers in *E. coli* and *P. aeruginosa*, there are also differences with respect to the mechanisms of generating antigenic variation and regulating pilin gene expression. Therefore, it is important to apply caution when attempting to make direct comparisons between two or more NMePhe operons from different genera. To gain a detailed understanding of the pilus operon of *P. aeruginosa*, it will be necessary to study it directly and avoid trying to infer mechanisms from other NMePhe systems too readily.

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The figure legend from page 16 has been removed due to difficulties in obtaining copyright permission. This page contained a figure legend explaining the diagram of the pilus structure on page 17. The figure legend was copied from:

Watts, T.H., Kay, C.M., Paranchych, W. (1983) Spectral properties of three quaternary arrangements of *Pseudomonas* pilin. Biochemistry 22: 3640-3646.

The figure from page 17 has been removed due to difficulties in obtaining copyright permission. This page contained a figure detailing the structure of a pilus. The figure was copied from:

Watts, T.H., Kay, C.M., Paranchych, W. (1983) Spectral properties of three quaternary arrangements of *Pseudomonas* pilin. Biochemistry 22: 3640-3646.

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Chapter II

CLONING AND SEQUENCING OF THE *PSEUDOMONAS AERUGINOSA* PAK PILIN GENE[1]

A. INTRODUCTION

Here we present the complete nucleotide sequence of a 1.2-kilobase *Hin*dIII restriction fragment from *P. aeruginosa* PAK encoding the entire pilin gene. The translated nucleotide sequence is compared to the published amino acid sequence for *P. aeruginosa* PAK pilin. We also present preliminary evidence that the pilin gene exists as a single copy within the chromosome.

B. MATERIALS and METHODS

Oligonucleotide probes of CA(A/G)TA(T/C)-CA(A/G)AA(T/C)TA(T/C)GT were purchased from New England Biolabs (MA) and end-labelled with T_4 polynucleotide kinase and (α-^{32}P)ATP (1). Southern blots (2) were prehybridized with 0.9 M NaCl, 0.09 M Tris (pH 7.5), 0.006 M EDTA, 0.5% sodium dodecyl-sulfate, 0.5 µg/ml sheared calf thymus DNA and 5 x Denhardt's solution [0.02% Ficoll, 0.02% bovine serum albumin,

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[1] A version of this chapter has been published. Pasloske, B.L., **Finlay, B.B.** and Paranchych, W. 1985. FEBS Lett. 183: 408-412.

0.02% polyvinylpolypyrrolidone in 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7)] at 34°C for 4 h, then hybridized under the same conditions with labelled synthetic oligonucleotides for 8 h. Blots were washed with 6 x SSC, 0.1% sodium dodecyl-sulfate at room temperature for 1 h and exposed to X-ray film. Double-stranded DNA was nick-translated and used to probe Southern blots as described in (3).

P. aeruginosa PAK chromosome was prepared using the procedure according to Coleman *et al.* (4). Complete *Hin*dIII restriction digests of *P. aeruginosa* PAK DNA were ligated into pUC8 and transformed into the *E. coli* strain JM83 (5). Colonies positive for inserts were plated onto nitrocellulose filters were washed free of cell debris (1) and then probed with the labelled synthetic oligonucleotides as above.

Recombinant plasmids were isolated in large quantities using the cleared lysate method described by Crosa and Falkow (6), followed by CsCl density centrifugation while smaller amounts were obtained by the method of Birnboim and Doly (7).

Nucleotide sequence analysis was performed using the Sanger dideoxy method (8) using M13 mp18/19 (9) or pUC 8 (10).

C. RESULTS and DISCUSSION

Several methods were utilized unsuccessfully in attempting to clone the *P. aeruginosa* PAK pilin gene. Whole cell immunoblot assays that were successful with the *Neisseria gonorrhoeae* pilin gene (11) did not work for *P. aeruginosa*. It is known that the first 30 amino acids at the amino terminus of *P. aeruginosa* PAK pilin and *N. gonorrhoeae* pilin are homologous (differing by 7 amino acids) (12). On this basis, it was predicted that the DNA encoding the *N. gonorrhoeae* pilin protein might share enough homology to permit its use as a probe for the *P. aeruginosa* pilin gene. A chimera containing the *N. gonorrhoeae* pilin gene (kindly provided by Dr. M. Koomey, Rockefeller University) was tested in this capacity. However, this probe failed to identify any homologous fragments with a Southern blot of *P. aeruginosa* chromosome, although it did identify four bands from a chromosomal *Eco*RI digest of a clinical isolate of *N. gonorrhoeae* (not shown).

Using the known *P. aeruginosa* PAK pilin sequence (13), a synthetic oligonucleotide was identified as a potential DNA probe for the pilin gene. The nucleotide sequence corresponding to residues 23 to 28 was chosen since it represented an area of low codon ambiguity. Several restriction digests of *P. aeruginosa* PAK chromosome were performed and a Southern blot of these digests made. When this blot was probed with the synthetic oligonucleotide pool and washed under low stringency conditions, a single, distinct band from each digest was found to contain homology in all cases (Fig. 1). Since a *Hin*dIII fragment of approximately 1.2 kilobases showed homology with the synthetic oligonucleotides, and because the *Hin*dIII digest contained few fragments in this size range (Fig. 1), a complete *Hin*dIII chromosomal digest was used for ligation into the vector pUC8. Chimeras containing inserts were tested by probing colony blots with the synthetic oligonucleotide pool. Five out of 250 colonies showed homology with this probe pool. DNA from one positive recombinant, pBP001, was isolated using a rapid plasmid procedure (7), digested with *Hin*dIII, and shown to contain a 1.2-kilobase fragment which had been inserted into pUC8. This DNA fragment was also sequenced using the oligonucleotide pool as a primer (8, 10) and shown to contain the N-terminal region of the *P. aeruginosa* PAK pilin.

The entire 1226-base-pair *Hin*dIII fragment was sequenced using the dideoxy method with M13 mp19 and pUC8 clones (Fig. 2). The complete *P. aeruginosa* PAK pilin gene was found within this *Hin*dIII fragment from nucleotides 459 to 908 (Fig. 3) and was the same as the published protein sequence (13), except that it contained a 6 amino acid leader sequence and a carboxy-terminal arginine, instead of lysine. This C-terminus variation is not an error in the protein sequence, but represents a real difference between pilin derived from a multi-piliated mutant of *P. aeruginosa* PAK, strain 2PfS, which was used for protein sequencing studies (13), and the wild-type strain of *P. aeruginosa* PAK, from which the present pilin gene was cloned.

According to the nucleotide sequence, the mature pilin molecule has 6 amino acids removed from its amino terminus. Most bacterial proteins which are to be secreted or inserted into the outer membrane have an amino-terminal extension which is subsequently removed (14). This signal sequence is usually characterized by a hydrophobic core of 12 to 20 amino acids with one or more positively charged amino acids preceding this region. The *P. aeruginosa* PAK pilin leader sequence lacks this hydrophobic core but the N-terminal region of the mature pilin is hydrophobic and could substitute for the hydrophobic region missing in the signal sequence. Four nucleotides (GAGG), 6 positions upstream of the ATG start codon of pilin (Fig. 3), match the consensus sequence for a ribosome binding site (15). The proposed translational start site for the PAK pilin gene is shown at nucleotide position 459. This was chosen in preference to position 447 (the only other possible start codon) because it was preceded by a potential ribosome binding site, while such a ribosome binding site was not evident in the region preceding position 447. The 1226-base-pair *Hin*dIII fragment also contains an open reading frame of 112 amino acids, extending from nucleotide 2 to 337 (Fig. 3). Perhaps this is a C-terminal portion of a protein found upstream of the pilin gene on the chromosome. No other reading frames of any significance were found in either direction on this fragment.

Other infectious organisms such as *Bacteroides nodosus* and *N. gonorrhoeae* have pilin proteins which have a homologous amino terminal region (16, 17). The nucleotide sequences for these pilin genes have also been determined (16, 17) and they have similar short, positively charged leader sequences with the last 3 residues of the leader conserved among all 3 leader sequences. The *P. aeruginosa* PAK leader sequence has two positive charges within 6 residues compared to one positive charge found within 7 residues in *N. gonorrhoeae* and *B. nodosus*. These charges may be important in preventing aggregation of pilin subunits before pilus assembly occurs.

Probing the same Southern blot as used for the synthetic oligonucleotides with the 610-base-pair *Pst*I fragment of the cloned DNA (containing the signal sequence and the first 96 amino acids) resulted in a blot identical to the one probed with the oligonucleotide pool (Fig. 1). With all digests only a single band appeared. These data indicate that the pilin gene is found as a single copy in the genome or within a very large repeated segment. This is in contrast with *N. gonorrhoeae*, which has at least 3 copies in its genome (17).

When the synthetic oligonucleotide pool was hybridized to various digests of *P. aeruginosa* PAO chromosomal DNA, no homology was detected, even though these proteins have similar amino acid sequences in the amino terminal region (Sastry, P. *et al.*, unpublished). It appears that the PAO pilin gene contains differences in the nucleotide sequence in the region of residues 23 to 28 which prevent the oligonucleotide pool from hybridizing. However, when the 610-base-pair *Pst*I fragment from PAK was used as a probe with PAO, a single band with detectable homology was found in all digests. We have cloned the *P. aeruginosa* PAO pilin gene and are currently determining its sequence.

FIGURE II.1

(A) 0.6% agarose gel of various chromosomal digests of *P. aeruginosa* PAK. (B) Autoradiograph of Southern blot of (A) hybridized with an oligonucleotide probe pool containing the sequences complementary to region of the pilin gene encoding amino acids 23 to 28 of the mature pilin (see Materials and Methods). Lanes: (1) λ/*Hin*dIII size standards in kilobases (from top to bottom) 23.7, 9.5, 6.7, 2.3, and 2.0, (2) *Eco*RI, (3) *Bam*HI, (4) *Sal*I, (5) *Pst*I, (6) *Hin*dIII, (7) *Eco*RI-*Bam*HI, (8) *Eco*RI-*Sal*I, (9) *Eco*RI-*Pst*I, (10) *Eco*RI-*Hin*dIII, (11) *Bam*HI-*Sal*I, (12) *Bam*HI-*Pst*I, (13) *Bam*HI-*Hin*dIII, (14) *Sal*I-*Pst*I, (15) *Sal*I-*Hin*dIII, (16) *Pst*I-*Hin*dIII.

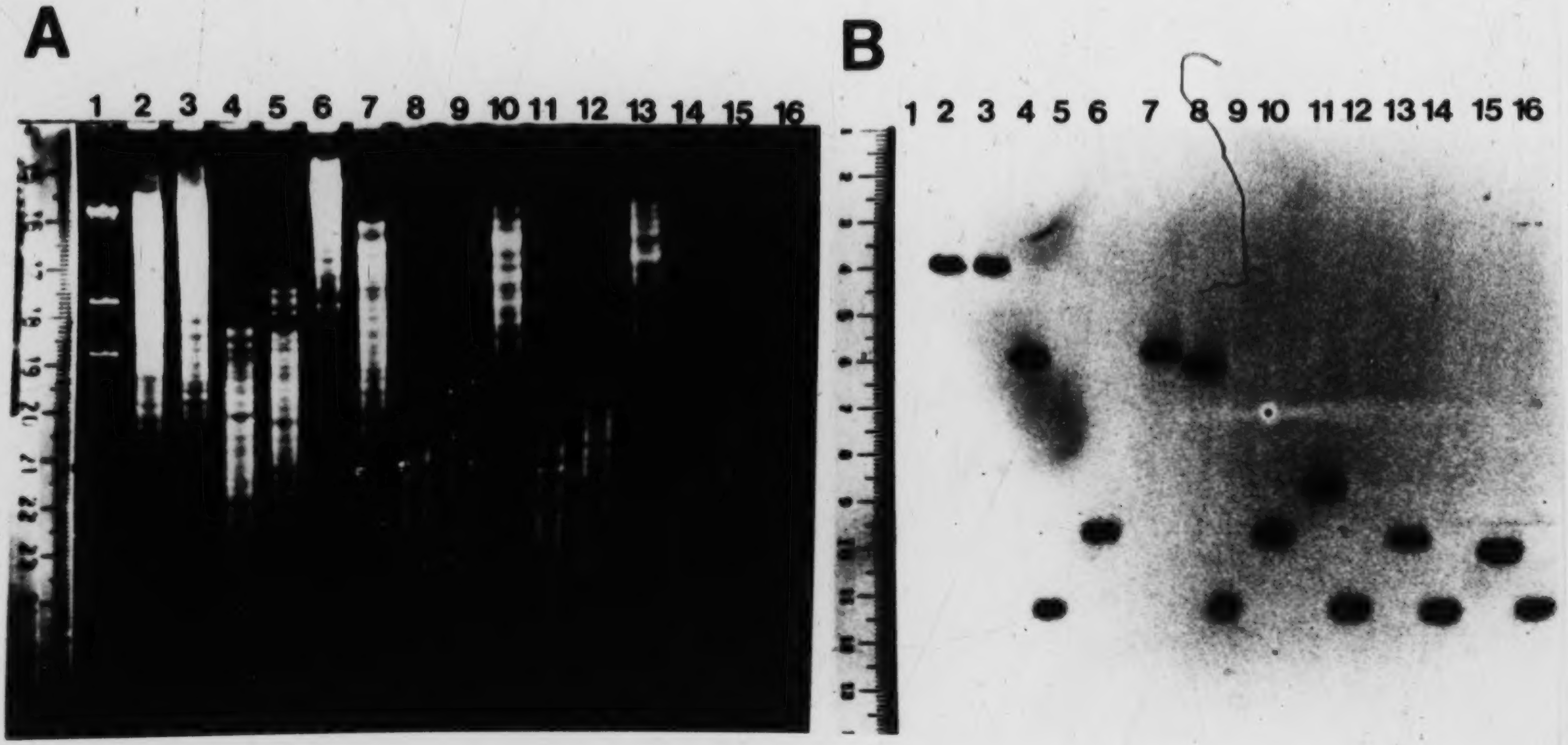
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIGURE II.2

Sequencing strategy for the 1.2-kilobase *Hin*dIII fragment (pBP001) containing the pilin gene. Bold arrows indicate location of pilin gene and direction of transcription. The direction of the sequenced DNA fragments is shown by arrows beneath the restriction map.

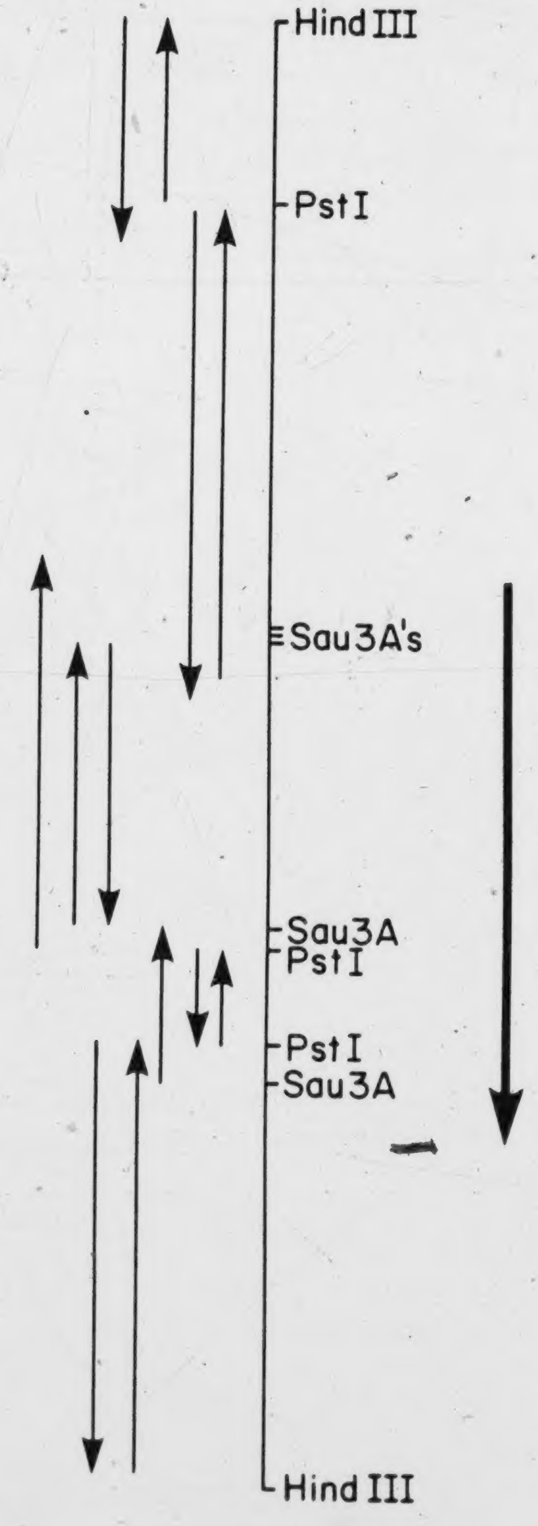
Hind III
Pst I
Sau3A's
Sau3A
Pst I
Pst I
Sau3A
Hind III

FIGURE II.3

Nucleotide sequence of the 1226-base-pair *Hin*dIII fragment (pBP001). The translated *P. aeruginosa* PAK pilin protein sequence is printed below the nucleotide sequence. Numbering below the protein sequence corresponds to the mature pilin protein. The proposed Shine-Dalgarno sequence is underlined.

```
                  20                  40                  60
AAGCTTTCCCTGTCCAGGCTGTTCAGGTCGCAATAGGCGATGCCGAACTGCTCGGCGGAC

                  80                 100                 120
AATTCGGCCAGGGCCAGGCCGCTTACCAGCTTGTTCTGCACCAGGTGCGTCACCAGCGAC

                 140                 160                 180
AGCTTGTTGCGCTGCGCCTGCGTCTGGGCCTGCAGGGCGGTCTTTTCGTCGAGTAGATTG

                 200                 220                 240
GCTTGGACGAGCTGTCGGGACAGACCGCTCAGTTGGATGCTGTCGTTCATGGAGAAGGAA

                 260                 280                 300
ATCGCAGAGGGCTATTGAAGTGCCTTATAACGCAGATAACAGGGTCTGCCAAATCGGGGG

                 320                 340                 360
AGTCCGGCTGTCAAAAAGTGTCACATCCTGTCCGTTTTAAGTTTGAGTCTCCTCGGGAAG

                 380                 400                 420
GGCATAAGACGGCCTTGCTCGCTATGTGAACGGTATTTGGCATGGTAAGTGCTTGGTAGG

                 440                 460                 480
GTTAGGCGTTAGGCCTATACATATCAATGGAGATATTCATGAAAGCTCAAAAAGGCTTTA
                                      MetLysAlaGlnLysGlyPheT
                                                          1
                 500                 520                 540
CCTTGATCGAACTGATGATCGTGGTTGCGATCATCGGTATCTTGGCTGCAATTGCCATTC
hrLeuIleGluLeuMetIleValValAlaIleIleGlyIleLeuAlaAlaIleAlaIleP
                        10                            20
                 560                 580                 600
CTCAGTATCAGAATTATGTAGCTCGTTCGGAAGGCGCATCTGCTCTTGCTTCGGTCAATC
roGlnTyrGlnAsnTyrValAlaArgSerGluGlyAlaSerAlaLeuAlaSerValAsnP
                        30                            40
                 620                 640                 660
CGTTGAAGACTACCGTTGAAGAGGCGCTTTCTCGTGGTTGGAGCGTGAAGAGCGGTACAG
roLeuLysThrThrValGluGluAlaLeuSerArgGlyTrpSerValLysSerGlyThrG
                        50                            60
                 680                 700                 720
GTACAGAGGACGCTACTAAGAAAGAGGTTCCTCTGGGGGTGGCGGCAGATGCTAACAAAC
lyThrGluAspAlaThrLysLysGluValProLeuGlyValAlaAlaAspAlaAsnLysL
                        70                            80
                 740                 760                 780
TGGGTACTATCGCACTCAAACCCGATCCTGCTGATGGTACTGCAGATATCACTTTGACTT
euGlyThrIleAlaLeuLysProAspProAlaAspGlyThrAlaAspIleThrLeuThrP
                        90                           100
                 800                 820                 840
TCACTATGGGCGGTGCAGGACCGAAGAATAAAGGCAAAATTATTACCCTGACTCGTACTG
heThrMetGlyGlyAlaGlyProLysAsnLysGlyLysIleIleThrLeuThrArgThrA
                       110                           120
                 860                 880                 900
CAGCTGATGGTCTCTGGAAGTGCACCAGTGATCAGGATGAGCAGTTTATTCCGAAAGCTT
laAlaAspGlyLeuTrpLysCysThrSerAspGlnAspGluGlnPheIleProLysGlyC
                       130                           140
                 920                 940                 960
GCTCTAGGTAATTTTGTAAGCGCTGGATGGTGTTGAACTATCGATGATTTGATGATAGTT
ysSerArgEnd
                 980                1000                1020
TCCACGCCGCCGCCGGATTAGCTCAGTCGGTAGAGCAGCTCATTCGTAATGAGAAGGTCG

                1040                1060                1080
GGGGTTCGATTCCTCTATCCGGCACCAGTCGCATAAAAAGCCCCGCTTCGGCGGGGCTTT

                1100                1120                1140
TTTTCGCCCTGCCGATTCGTTCAGAGGGTGAGGCGCATGGACAGGTCGACGGCCCTGACGTC

                1160                1180                1200
CTTGGTCAGGGTGCCCGATGCAGATGTAGTCGACGCCCGGTCTCGGCCGATGTTGCCGCAGGCT

                1220
GCTTTCGTTGATCCCGCCGGAAGCTT
```

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Chapter III

TWO UNUSUAL PILIN SEQUENCES FROM DIFFERENT ISOLATES OF *PSEUDOMONAS AERUGINOSA*[1]

A. INTRODUCTION

The DNA sequences and the predicted primary structures are predicted for two other *P. aeruginosa* NMePhe pilins which have several different features compared to any other published *P. aeruginosa* pilin sequence.

B. MATERIALS and METHODS

P. aeruginosa strain P1 was originally cultured from the sputum of a patient with cystic fibrosis in the University of Minnesota Hospital, Minneapolis (18). It was generously provided by D.P. Speert, B.C. Children's Hospital Research Centre, Vancouver, B.C., Canada. *P. aeruginosa* strain K122-4 was isolated from a cystic fibrosis patient in Toronto and was provided by R.T. Irvin, Department of Botany and Microbiology, Erindale College, University of Toronto, Missisauga, Ontario, Canada.

The chromosomal DNA from these two strains was isolated, subjected to several restriction enzyme digestions, fractionated

[1] A version of this has been published. **Pasloske, B.L., Sastry, P.A., Finlay, B.B., Paranchych, W.** 1988. J. Bacteriol. 170: 3738-3741.

and blotted onto nitrocellulose filter paper as previously published (18). The blots were allowed to hybridize to to the nick translated 610-base-pair *Pst*I fragment from strain PAK which codes for the first 95 amino acids of mature pilin and also has about 320 base pairs of DNA upstream of the pilin gene (14). High stringency hybridization conditions were used and the washes were performed at room temperature followed by subjecting the blots to autoradiography (9).

Interestingly, strain P1 was the first isolate which did not contain the pilin gene in a 1.2-kilobase (kb) *Hin*dIII fragment. The *Hin*dIII fragment was greater than 21 kb and therefore not useful for cloning and sequencing. Instead, a 3.5-kb *Hin*dIII-*Bam*HI fragment was cloned into the vector pUC19 (chimera pP1-001) by shotgun cloning methodology (14). *Escherichia coli* DH5α (K-12 F^- *endA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1* λ^- *recA1 gyrA96 relA1* φ80d*lacZ*ΔM15) was purchased from Bethesda Research Laboratories, Burlington, Ontario, Canada, and was the strain used for DNA transformations. A 1.5-kb *Sph*I-*Pst*I fragment containing the entire pilin gene was subcloned into pUC19 (chimera pP1-005) using *E. coli* JM109 (19) for the transformations. A 730-base-pair *Sph*I fragment directly upstream of the *Sph*I-*Pst*I fragment was subcloned from pP1-001 into pUC19 to create pP1-006. This clone was constructed to obtain sequence information upstream of the P1 pilin gene.

On the other hand, strain K122-4 had a 1.2-kb *Hin*dIII fragment which hybridized to the PAK probe. The shotgun strategy used to clone the P1 pilin gene was also used to clone the 1.2-kb *Hin*dIII fragment using the vector pUC19 (chimera pK122-4) and *E. coli* JM109.

The recombinant plasmids pP1-005, pP1-006, and pK122-4 were isolated in large quantities by CsCl density centrifugation and the pilin genes were sequenced using the Sanger dideoxy method (14). Both strands of both pilin genes were sequenced. The enzymes *Sau*3A and *Sph*I were used to subclone the P1 pilin gene and two synthetic oligonucleotides encompassing nucleotide positions 263 to 279 and 664 to 680 (Fig. 1) were used as primers to obtain overlaps and the sequence of the opposite strand. *Alu*I, *Sau*3A, and *Hin*dIII were the only restriction enzymes required to subclone the K122-4 pilin gene for the complete sequence.

C. RESULTS and DISCUSSION

The DNA sequences along with the predicted protein sequences of the P1 and K122-4 pilin genes are shown in Fig. 1. Within the two DNA sequences, starting at approximately nucleotide position 120, are the predicted NtrA-dependent promoters as previously recognized by Johnson *et al.* (8). The putative NtrA-dependent promoter is a region 10 to 26 base pairs upstream of the transcription start site, where the *ntrA* gene product, acting as a sigma factor, is believed to be required for transcription initiation. *Pseudomonas* NtrA-dependent promoters have also been discovered for the *xylABC* operon of the TOL plasmid (5) and the carboxypeptidase G2 gene (11) of *Pseudomonas putida*. In many enteric bacteria, genes involved in nitrogen assimilation, such as *glnA*, as well as the nitrogen fixation genes in organisms like *Klebsiella pneumoniae*, have NtrA-dependent promoters, and these genes are regulated by the gene products encoded by *ntrC* and *nifA*, respectively (6). NtrC and NifA both recognize specific sequences upstream of the genes that they positively regulate. Analysis of the DNA upstream of the pilin genes from *P. aeruginosa* strains PAK (16), PAO (16), PA103 (8), P1, and K122-4 revealed a conserved sequence among all five strains which was located approximately 114 nucleotides upstream of their putative transcription initiation sites. This sequence had a strong homology with the NifA recognition sites of the nitrogen fixation genes which are normally located 100 to 150 nucleotides upstream of the transcription initiation site (Fig. 1) (3). Table 1 outlines the hypothetical NifA recognitions sites associated with each pilin gene. These sequences are almost identical to the NifA-binding site consensus sequence of TGT-N_{10}-ACA (3) except for the difference in the spacing between the conserved trinucleotides such that N equals 11 instead of 10. Since *P. aeruginosa* is not known to have a *nifA* gene, an *ntrC*-type regulatory gene may be responsible for this regulation. Pilin expression and nitrogen assimilation (7) in *P. aeruginosa* may both be regulated by an NtrC-like protein which associates with the NifA-like recognition site since the *ntrC* and *nifA* genes are often interchangable for gene activation (6).

In Fig. 2, the predicted amino acid sequences of the five known *P. aeruginosa* pilins have been aligned to obtain the best homology possible. There are some features in the K122-4 and P1 pilins which make them distinctly different from the other published pilins. Both of these pilin sequences diverge from each other and the other pilins at amino acid position 31 while pilin sequences of strains PAK, PAO, and CD differ after position 55. This point of divergence at position 31 is similar to that of the NMePhe pilins of other genera (4). The strain P1 pilin is different from the other four pilins in that it contains the largest putative C-terminal disulfide loop, which contains 17 residues compared with 12 in other strains.

The K122-4 pilin has three characteristics which make it unusual: (i) it has 7 residues instead of 6 as a putative leader peptide; (ii) it is the longest NMePhe pilin found in *P. aeruginosa* to date, with 150 residues in its putative mature form; and (iii) it has two additional cysteines at positions 58 and 100. Similarily situated cysteines within the pilin of *B. nodosus* strain 198 were shown to be in the reduced state (10), suggesting that the analogous cysteines of K122-4 pilin may also be in the reduced state.

It is worth noting that certain amino acids besides the hydrophobic stretch at the N terminus appear to be conserved in all 5 sequences: Leu-43; Lys-44, and -86; Thr-45, -65, -106, and -124; Gly-55, -99, and -133; Trp-137; Cys-139, and -157; Pro-154; and the aromatic amino acids at position 152. These residues may be structurally important for pilus assembly or for the maintenance of the receptor-binding domain directed towards epithelial tissues.

The pilins of strains PA103 (8) and PA1244 (J. Sadoff, personal communication) have almost identical sequences to the CD (15) and P1 pilins, respectively. Moreover, we have sequenced other isolates with pilins nearly identical to those of strains PAK and K122-4. Interestingly, the strains which had closely related pilin sequences were isolated from patients in widely separated locations throughout North America. These data suggest that the number of unique pilin types may be fairly limited, perhaps 10 to 15. This number would be in keeping with *B. nodosus* (1) and *M. bovis* (12), in which there are less than 10 known pilus serogroups for each pathogen.

The predicted surface domains for each of the five pilins were determined using the program of Parker *et al.* (13). These surface plots were studied to locate regions where the surface residues of all 5 pilins overlapped (Fig. 2). The surface epitopes predicted on this basis were the residues at positions 22 to 34 (Fig. 2, domain I), 44 to 48 (II), 64 to 72 (III), 79 to 87 (IV), 95 to 100 (V), 110 to 117 (VI), 127 to 131 (VII), 142 to 150 (VIII), and 155 to the C-terminus (IX). It is of interest that the strongest predicted surface epitopes (domains III, V, VI and VIII) were in similar regions for all five pilins despite the variations in the amino acid sequences. Domains III and V, located within the central, hypervariable region of the pilins, fall within the antigenic determinants predicted by a sequence comparison of strains PAK, PAO, and CD (15) and experimental data gathered from pilin peptides confirm these predictions as well (17; Sastry and Paranchych, unpublished results).

FIGURE III.1

The nucleotide sequences and the predicted amino acid sequences of the pilins from *P. aeruginosa* P1 and K122-4. The nucleotides are numbered along the top of the sequences, and the amino acids are numbered along the bottom of the sequences. Negative numbering indicates the amino acid positions within the putative leader peptides of the two pilins. The arrows indicate the transcription initiation sites as predicted by homology comparison with the previously determined transcription initiations sites of the PAK pilin gene (8). Single-underlined sequences are the consensus sequences for the putative NtrA-dependent promoters (positions 119 to 133 for K122-4 and positions 118 to 132 for P1) (8). Double-underlined regions indicate the putative NifA recognition sites (positions 29 to 45 for K122-4 and 28 to 44 for P1) (6). The predicted cysteine residues of the two pilins are boxed.

K122-4

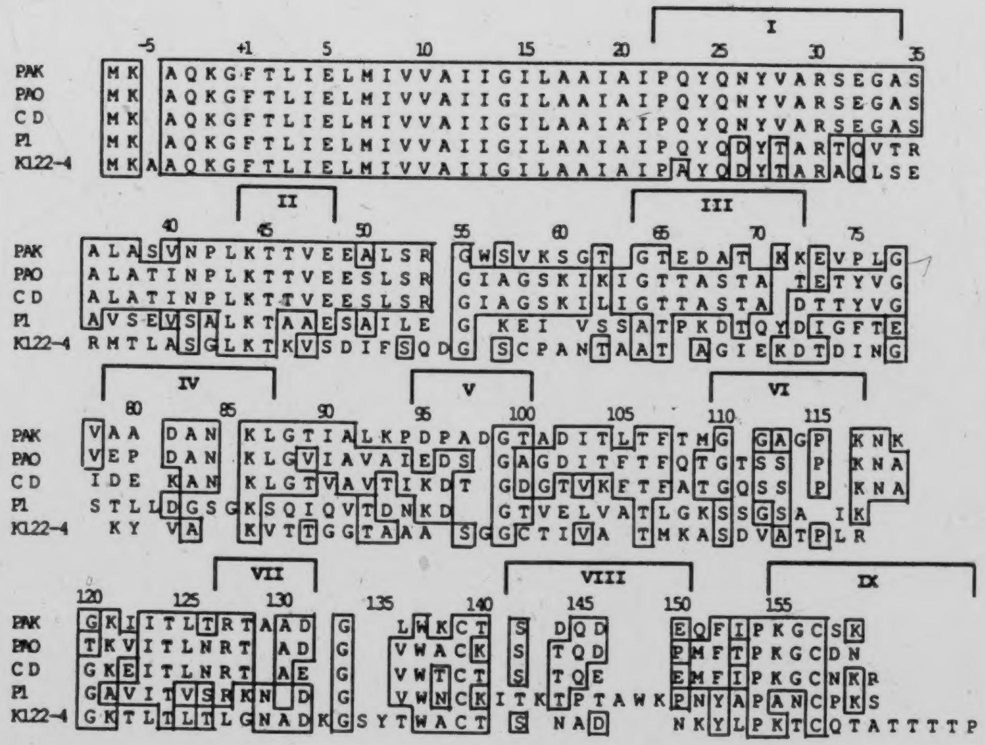
```
                  20                  40                  60
AGGGGCTGCCAAATCGAGGAAATCCAGCTGTCAAAAAATGTCACATCCTGTCCGTTTTAA

                  80                 100                 120
GTTTGACTCTCATCGAGAAAGGAGCCCAGTTTCCTTGATCATGGCACGGTTTGATATTTG

                 140                 160                 180
GCATGGTAAGTGCTTGTTGAGGATCAGGCGTTAGGCCTATACATATCAATGGAGAAATTC
                        ↑
                 200                 220                 240
ATGAAAGCTGCTCAAAAAGGCTTTACCTTGATCGAATTGATGATCGTGGTCGCGATCATC
MetLysAlaAlaGlnLysGlyPheThrLeuIleGluLeuMetIleValValAlaIleIle
 -7                  +1                          +10

                 260                 280                 300
GGTATTCTGGCTGCCATCGCTATTCCAGCGTATCAGGACTACACCGCACGCGCTCAGCTT
GlyIleLeuAlaAlaIleAlaIleProAlaTyrGlnAspTyrThrAlaArgAlaGlnLeu
                 +20                             +30

                 320                 340                 360
AGCGAACGCATGACCCTGGCCAGTGGTCTCAAGACGAAAGTGAGCGATATCTTCTCTCAG
SerGluArgMetThrLeuAlaSerGlyLeuLysThrLysValSerAspIlePheSerGln
                 +40                             +50

                 380                 400                 420
GATGGGTCCTGCCCGGCTAATACTGCTGCCACGGCAGGCATCGAGAAAGATACCGACATC
AspGlySerCysProAlaAsnThrAlaAlaThrAlaGlyIleGluLysAspThrAspIle
                 +60                             +70

                 440                 460                 480
AACGGCAAGTATGTTGCCAAGGTAACAACTGGTGGCACCGCAGCTGCGTCTGGTGGTTGC
AsnGlyLysTyrValAlaLysValThrThrGlyGlyThrAlaAlaAlaSerGlyGlyCys
                 +80                             +90

                 500                 520                 540
ACTATCGTTGCTACTATGAAAGCCTCTGATGTGGCTACTCCTCTGAGGGGGAAAACTCTG
ThrIleValAlaThrMetLysAlaSerAspValAlaThrProLeuArgGlyLysThrLeu
                +100                            +110

                 560                 580                 600
ACTTTGACTCTAGGAAATGCTGACAAGGGTTCTTACACTTGGGCCTGTACTTCCAACGCA
ThrLeuThrLeuGlyAsnAlaAspLysGlySerTyrThrTrpAlaCysThrSerAsnAla
                +120                            +130

                 620                 640                 660
GATAACAAGTACCTGCCAAAAACCTGCCAGACTGCTACCACTACCACTCCGTAATAAAGT
AspAsnLysTyrLeuProLysThrCysGlnThrAlaThrThrThrThrPro
                +140                            +150

                 680
AGCATACGGCTAGGTTGTGCCGGATTAG
```

P1

```
                  20                  40                  60
GGGGCTGCCAAACCGACGATTCCGAGATGCCAAAAAGTGTCACATATTGCCGGCAGGGGA

                  80                 100                 120
GAGCGGAACACTTTCGAAGTCCCCGCAAGAGTTGCGAAAGGAGGGCGTTTGAAAGGTTGG

                 140                 160                 180
CATGCAACCTGCTTTGAAGGGGGAAGGCGTTTGGCCTATACATACCACATGGAGATATTC
                      ↑
                 200                 220                 240
ATGAAAGCTCAGAAGGGTTTTACTCTGATCGAACTGATGATCGTGGTTGCGATCATCGGC
MetLysAlaGlnLysGlyPheThrLeuIleGluLeuMetIleValValAlaIleIleGly
 -6                  +1                          +10

                 260                 280                 300
ATCCTGGCCGCCATTGCCATCCCGCAATACCAGGACTACACCGCCCGTACCCAGGTGACC
IleLeuAlaAlaIleAlaIleProGlnTyrGlnAspTyrThrAlaArgThrGlnValThr
              +20                             +30

                 320                 340                 360
CGTGCCGTGAGTGAAGTCAGCGCGCTGAAGACCGCTGCGGAGTCGGCGATTCTGGAAGGG
ArgAlaValSerGluValSerAlaLeuLysThrAlaAlaGluSerAlaIleLeuGluGly
              +40                             +50

                 380                 400                 420
AAGGAGATTGTTTCCAGCGCGACTCCTAAAGATACCCAGTATGACATTGGCTTCACCGAG
LysGluIleValSerSerAlaThrProLysAspThrGlnTyrAspIleGlyPheThrGlu
              +60                             +70

                 440                 460                 480
TCTACTTTGCTAGATGGTTCTGGTAAGAGTCAGATCCAGGTAACGGACAATAAAGATGGC
SerThrLeuLeuAspGlySerGlyLysSerGlnIleGlnValThrAspAsnLysAspGly
              +80                             +90

                 500                 520                 540
ACCGTTGAGTTGGTCGCTACCTTGGGTAAATCTTCTGGTTCCGCCATCAAAGGGGCTGTA
ThrValGluLeuValAlaThrLeuGlyLysSerSerGlySerAlaIleLysGlyAlaVal
             +100                            +110

                 560                 580                 600
ATCACTGTTTCGCGTAAAAATGACGGAGTCTGGAACTGCAAAATCACCAAAACTCCTACA
IleThrValSerArgLysAsnAspGlyValTrpAsnCysLysIleThrLysThrProThr
             +120                            +130

                 620                 640                 660
GCTTGGAAGCCCAACTACGCTCCGGCTAATTGCCCGAAATCCTAATCGGTTTTTTGAGTT
AlaTrpLysProAsnTyrAlaProAlaAsnCysProLysSer
             +140

                 680
GTTTTGAGTGTGAAGGAAGCTCCCCGATT
```

FIGURE III.2

Comparison of the five pilin sequences of *P. aeruginosa* strains PAK/2PfS (16), PAO (16), CD (15), P1, and K122-4 and their predicted surface domains. The pilin sequences were aligned to obtain the greatest possible homology by visual examination on the basis of complete identity and by using the amino acid similarity values of Bacon and Anderson (2). Empty spaces were allowed for best possible alignment and positions occupied by identical amino acids are boxed. The numbering of the amino acids was based on the total number of positions including spaces rather than using the numbering of any particular pilin as a standard. The overlined sequences indicate the predicted surface domains I through IX (see text) as determined by the program of Parker *et al.* (13).



```
                                          I
         -5    +1  5    10   15   20   25   30   35
PAK     MK AQKGFTLIELMIVVAIIGILAAIAIPQYQNYVARSEGAS
PAO     MK AQKGFTLIELMIVVAIIGILAAIAIPQYQNYVARSEGAS
CD      MK AQKGFTLIELMIVVAIIGILAAIAIPQYQNYVARSEGAS
P1      MK AQKGFTLIELMIVVAIIGILAAIAIPQYQDYTARTQVTR
K122-4  MKAAQKGFTLIELMIVVAIIGILAAIAIPAYQDYTARAQLSE

                  II                    III
            40   45   50   55   60   65   70   75
PAK     ALASVNPLKTTVEEALSR GWSVKSGT GTEDAT KKEVPLG
PAO     ALATINPLKTTVEESLSR GIAGSKIKIGTTASTA TETYVG
CD      ALATINPLKTTVEESLSR GIAGSKILIGTTASTA DTTYVG
P1      AVSEVSALKTAAESAILE G KEI VSSATPKDTQYDIGFTE
K122-4  RMTLASGLKTKVSDIFSQDG SCPANTAAT AGIEKDTDING

            IV             V               VI
          80   85   90   95   100  105  110  115
PAK     VAA DAN KLGTIALKPDPADGTADITLTFTMG GAGP KNK
PAO     VEP DAN KLGVIAVAIEDS GAGDITFTFQTGTSS P KNA
CD      IDE KAN KLGTVAVTIKDT GDGTVKFTFATGQSS P KNA
P1      STLLDGSGKSQIQVTDNKD  GTVELVATLGKSSGSA IK
K122-4   KY VA  KVTTGGTAAA SGGCTIVA TMKASDVATPLR

                 VII              VIII         IX
        120  125  130  135  140  145  150  155
PAK     GKIITLTRTAAD G  LWKCT S DQD   EQFIPKGCSK
PAO     TKVITLNRT AD G  VWACK S TQD   PMFTPKGCDN
CD      GKEITLNRT AE G  VWTCT S TQE   EMFIPKGCNKR
P1      GAVITVSRKN D G  VWNCKITKTPTAWKPNYAPANCPKS
K122-4  GKTLTLTLGNADKGSYTWACT S NAD   NKYLPKTCQTATTTTP
```

TABLE III.1

Putative NifA Recognition Sites from the Pilin Genes of Five *P. aeruginosa* Isolates.

Strain (reference)	Sequence[a]	Position of Consensus G[b]
Consensus(3)	TGT...........ACA	-100 to -150
PAK(16)	GGAGTCCGGCTGTCAAAAAGTGTCACATCCTGTCCGT	-113
PAO(16)	GAAATCCAGCTGTCAAAAAATGTCACATCCTGTCCGT	-114
PA103(8)	GAAATCCAGCTGTCAAAAAATGTCACATCCTGTCCGT	-114
K122-4	GAAATCCAGCTGTCAAAAAATGTCACATCCTGTCCGT	-114
P1	GATTCCGACATGCCAAAAAGTGTCACATATTGCCGGC	-114

a. Underlined nucleotides indicate differences from the predominant nucleotide of that position.

b. Relative to the putative transcriptional initiation sites at positions 143 and 144 for P1 and K122-4, respectively (Fig. 1).

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Chapter IV

EXPRESSION OF *PSEUDOMONAS AERUGINOSA* PAK PILIN GENE IN *ESCHERICHIA COLI*[1]

A. INTRODUCTION

Several pathogenic gram-negative organisms have chromosomally encoded NMePhe adherence pili. These include *Neiserria gonorrhoeae* (15, 16), *Bacteroides nodosus* (7), *Moraxella nonliquefaciens* (9), *Moraxella bovis* (13) and *Pseudomonas aeruginosa* PAK (18, 22). Pilin from these organisms have a highly conserved hydrophobic amino terminus (residues 1 to 28) as well as a short (6- to 7-amino acid) positively charged leader sequence. The remaining residues in these pilin proteins appear unrelated. Since these pathogenic organisms all share leader peptide and amino terminus homology, it is important to examine the expression and transport mechanisms of *P. aeruginosa* PAK pilin, since these diverse pilus proteins may share similar transport pathways.

With this goal in mind, we present evidence of expression of the *P. aeruginosa* PAK pilin gene in *E. coli*. Experiments presented here include an assay of pilin promoter strength and studies on the compartmentalization and processing (or lack thereof) of the pilin gene product.

[1] A version of this paper has been published. **Finlay, B.B., Pasloske, B.L. and Paranchych, W.** 1986. J. Bacteriol. 165: 625-630.

B. MATERIALS and METHODS

1. Bacterial Strains and Plasmids.

The bacterial strains used were wild type *Pseudomonas aeruginosa* PAK (a gift from D. E. Bradley, Memorial University, Saint John's, Newfoundland), *Escherichia coli* JM83 (25), *E. coli* P67854 (1), *E. coli* JC5363 (14) *E. coli* HB101 (3), and *E. coli* JE2571 (Fla$^-$). The plasmids used were pUC8 (25) and pBP001 (18), a chimera of a 1.2-kilobase *Hind*III fragment containing the *P. aeruginosa* PAK pilin gene in pUC8. pBP010 is the *Hind*III-*Sau*3A 482-base-pair fragment from pBP001 upstream of the PAK pilin gene.

2. Isolation and Labeling of Minicells.

A culture of strain P67854 (1) containing the desired plasmid was grown to stationary phase, and minicells were purified essentially as described by Roozen *et al.* (20), except whole cells were removed by pelleting for 4 minutes at 1500 x *g* before loading onto sucrose gradients. [^{35}S]methionine (50 µCi) of was used to label minicells.

3. SDS-PAGE.

Sodium dodecyl-sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) was done with 15% SDS-polyacrylamide gels which were prepared as described by Laemmli (11). The gels were soaked in water (1 h) and then in 1 M sodium salicylate (1 h) before vacuum drying and autoradiography were performed.

4. Minicell Fractionation.

Labeled minicells were washed in 20% sucrose-30 mM Tris hydrochloride (pH 8) and resuspended in 1 ml 50 mM Tris hydrochloride (pH 7.5)-100 μg of gelatin per ml. Next, 40 μl of 0.25 M EDTA-5 mg of lysozyme per ml was added and incubated at 37° for 1 h. Spheroplasts were removed by centrifuging for 3 min in an Eppendorf centifuge. The supernatant solution (periplasmic fraction) was precipitated with 10% trichloroacetic acid (TCA), while the pellet (spheroplasts) was resuspended in 50 mM potassium phosphate buffer (pH 6.6), containing 10 μg each of DNAse and RNAse per ml and sonicated with five 1-min pulses at 0°C. Unsonicated cells were removed after the initial 3-min centrifugation, and the membranes were pelleted (165,000 x *g* at 0°C for 1 h). The supernatant solution (cytoplasmic fraction) was precipitated in 10% trichloroacetic acid. The pellet (membrane fraction) was resuspended in cold potassium phosphate buffer. All fractions were then counted in a Beckman LS6800 scintillation spectrometer and subjected to SDS-PAGE and autoradiography.

5. Whole Cell Fractionation.

The inner and outer membrane fractions of *E. coli* HB101 (pBP001) were separated based on the procedure by Yamato *et al.* (27), except that the dialysis step was omitted and the inner membrane fraction was purified twice by layering on 44% (wt/wt) sucrose-3 mM EDTA (pH 7.2) and centrifuging at 176,000 x *g* for

60 min. After each centrifugation through the sucrose, the inner membrane fraction was diluted threefold in 3mM EDTA (pH 7.2), pelleted, and resuspended in 10% (wt/vol) sucrose-3 mM EDTA (pH 7.2). Periplasmic proteins were purified using the cold shock procedure desribed by Dougan *et al.* (6).

To obtain the cytoplasmic proteins, spheroplasts were prepared from 500 ml of cells by the cold-shock technique (6) and then resuspended in 8 ml of cold water. The cells were passed through a French pressure cell three times at 14,000 lb/in^2. The unlysed cells were removed by centrifugation at 8,000 x *g* for 15 min. The supernatant was decanted into a Beckman SW41 centrifuge tube, topped with cold distilled water and centrifuged 3 h at 39,000 rpm to remove the crude membrane fraction. The supernatant was dried in a rotoevaporator and then subjected to SDS-PAGE.

6. Preadsorption of Antiserum.

A 40-ml culture of *E. coli* HB101 was grown to late exponential phase and resuspended in 25 ml of the buffer used in the immunoblot procedure. To inhibit protease activity, 62 µl of 40 mM phenylmethylsulfonyl fluoride in 95% ethanol was added to the cell suspension. The cells were sonicated with five 1.5-min pulses of the Braunsonic 1510 sonicator at 4°C with a small probe. After lysis, 80 µl of the anti-PAK pili serum was added, the mixture was incubated at 4°C for 24 h with gentle rotation and then used in the immunoblot procedure.

7. Galactokinase K Assay.

This assay was performed as described previously (14), except that the specific activity of undiluted [^{14}C]galactose was 59.6 mCi/mmol. Units are expressed as nanomoles of galactose phosphorylated per minute at an optical density of 650 nm.

C. RESULTS

1. Expression of PAK Pilin in *E. coli* Minicells.

A chimera containing the *P. aeruginosa* PAK pilin gene in the vector pUC8, called pBP001 (18), was transformed into the *E. coli* minicell strain P67854. ^{35}S-labeled minicells were harvested and subjected to SDS-PAGE followed by autoradiography. The results of such an experiment with pBP001 and pUC8 are shown in Fig. 1A. pBP001 makes only one novel protein, unlike pUC8. This heavily expressed protein has a molecular weight of 15,600 and migrates slightly slower than *P. aeruginosa* PAK pilin (15,000 daltons). We hypothesized that this protein was unprocessed PAK pilin with a six-amino-acid (660-dalton) leader sequence (18). (The addition of 2-phenylethyl alcohol, a known inhibitor of signal peptide cleavage, had no effect on mobility of this gene product [data not shown].) To test this hypothesis, unlabeled minicells were harvested, electrophoresed and subjected to immunoblotting using unpreadsorbed anti-PAK pili serum (Fig. 1B). The 15,600-dalton protein and PAK pilin were both recognized by anti-pili serum, suggesting that this protein is probably unprocessed PAK pilin. Several fainter bands were also visible with both pBP001 and pUC8, suggesting that antibodies to several *E. coli* antigens were present in the antiserum.

2. Cellular Location of PAK Pilin in *E. coli* Minicells.

Besides being present in assembled pili, *P. aeruginosa* pilin is present in both the inner and outer membranes (26). To determine the cellular location within *E. coli* minicells, labeled minicells containing pBP001 were fractionated as described above, and 30,000-cpm fractions were separated by SDS-PAGE and autoradiographed (Fig. 2). Unprocessed PAK pilin was distributed in the cytoplasm, and in the membrane fraction. Only a small amount of unprocessed pilin was visible in the periplasmic fraction but comparison of the amounts of pilin found in each fraction was not possible as an equal amount of radioactivity, not equivalent amounts of each cellular fraction, was loaded for each fraction. There was no evidence of processed pilin in any fraction, suggesting that transport of pilin within minicells to the membrane of *E. coli* is independent of leader sequence removal. Moreover, attempts to detect pilin in culture media after the labeling of cells with [^{35}S]methionine, by means of trichloroacetic acid precipitation of the culture medium and SDS-PAGE examination of the acid precipitate showed that pilin is not secreted extracellularly (data not shown).

3. Cellular Location of PAK Pilin in *E. coli*.

To determine the cellular location and distribution of PAK pilin in whole cells, *E. coli* HB101 (pBP001) was fractionated as described above. Approximately equivalent amounts of each cellular fraction was separated on a 15% polyacrylamide gel and PAK pilin was detected using an immunoblot procedure with anti-PAK pili serum preadsorbed against HB101 (Fig. 3). This analysis revealed that the majority of the pilin antigen was found in the inner membrane fraction (lane 5). *M. bovis* pilin, which has a similar leader sequence and amino terminus, was also found associated with the inner membrane fraction of HB101 (13).

The inner membrane fraction of HB101 (pBP001) also contained a fainter band which migrated slightly above the pilin band. We suggest that this band is prepilin, as it had the correct predicted molecular mass and reacted with anti-pili serum. The presence of prepilin in the membrane fraction from minicells and in the inner membrane fraction from HB101 suggests that proteolytic removal of the six-residue leader peptide is not required for transport of pilin to the inner membrane.

Although pBP001 encodes only one complete polypeptide, PAK pilin (18), tests were performed to determine whether an intact pilus was present and functional. Bacteriophage spot tests with the pilus-specific bacteriophage PO4 were negative. When pBP001 was transformed into the bald *E. coli* JE2571 (JM83 expresses type 1 pili) and examined in the electron microscope, no pili were visible (data not shown).

Collectively, these data demonstrate that the chimera containing the *P. aeruginosa* PAK pilin gene expresses unprocessed and processed pilin which is located predominantly in the inner membrane of *E. coli* and not assembled into pili.

4. Promoter Studies.

Sequencing data of pBP001 demonstrated that the pilin gene was located in the opposite orientation of the pUC8 *lac* promoter (unpublished). This suggested that pBP001 had its own promoter, which was being utilized in *E. coli*. To test for the presence of a promoter upstream of the pilin gene, the 484-nucleotide *Hind*III-*Sau*3A fragment was inserted into the promoter assessment vector pKO-4 (14). This fragment contains the entire region upstream of the pilin gene, as well as DNA encoding the first nine amino acids of unprocessed pilin. pKO-4 contains the structural gene for galactokinase K (*galK*) but is constructed such that there is no promoter upstream from the *galK* gene. Unique *Hind*III and *Bam*HI sites upstream of the *galK* gene allow insertion of DNA fragments and measurement of promoter strength therein. Chimeras containing the 484-base-pair fragment within pKO-4 demonstrated *galK* activity on MacConkey agar-galactose plates. The results of a *galK* assay (14) of this chimera indicate that, compared with the background of pKO-4 (1.63 U [nmol of galactose phosphorylated per min at A_{650}]), the pBP010 with the PAK pilin promoter contained about 1.5 times as much promoter activity (2.79 U). Both of these activities were very low as compared with that of the *lac* promoter assayed in pKL200 (56.8 U); nonetheless, they suggest that a weak promoter does exist within the first 484 nucleotides of pBP001. It is of interest that the pilin gene is expressed efficiently despite the weak promoter activity. This suggests that the pilin, the mRNA, or both may be exceptionally stable within the *E. coli* cells.

D. DISCUSSION

Several *P. aeruginosa* genes are expressed in *E. coli*. Phospholipase C (hemolysin), normally a secreted protein in *P. aeruginosa*, was reported to be in the cytoplasm (5, 24) and in the outer membrane (12) when expressed in *E. coli*. A vector promoter was required for production of this protein within *E. coli* (24). Exotoxin A, another secreted protein of *P. aeruginosa*, was also found to be expressed in *E. coli* (10). This protein was unprocessed, was found mainly in the cytoplasm, and required a vector promoter for expression in *E. coli*. Recently, phosphomannose isomerase, a cytoplasmic protein from *P. aeruginosa*, was reported to be expressed in *E. coli* when placed under vector promoter control (A. Darzins *et al*., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985,, D94, p. 70). Other examples of *Pseudomonas* species with promoters that are not recognized in *E. coli* include *P. fluorescens* (4) and *P. putida* (17). Thus, it seems that *Pseudomonas* promoters are not readily utilized in *E. coli*. We have reported here that a putative promoter for the *P. aeruginosa* PAK pilin gene appears to be recognized in *E. coli*. Examination of the first 450 bases of pBP001 revealed several sequences that partially resemble the consensus sequence for the *E. coli* promoter (21); however, we were unable to determine which of these may be serving as the promoter for the pilin gene.

The *P. aeruginosa* phospholipase C and exotoxin A gene products are unable to undergo signal peptide processing or secretion into the medium when expressed in *E. coli* (5, 12, 24). Normal processing of the PAK pilin involves the cleavage of a six-residue leader peptide and transport to the inner and outer membranes (18, 26). We have shown that this leader is not always removed in *E. coli* but that both pilin and prepilin are transported to the inner membrane.

The observed interactions of PAK pilin with *E. coli* membranes may be considered in terms of the "helical hairpin hypothesis" proposed by Engelman and Steitz (8). These workers suggested that during synthesis of a membrane protein, approximately 40 to 50 amino acid residues of the nascent polypeptide chain fold in an aqueous environment to form an antiparallel pair of helices consisting of a hydrophobic leader peptide containing 15 to 20 amino acid residues and a similar length of the polypeptide immediately distal to the hydrophobic leader region. The helical hairpin thus formed inserts spontaneously into the hydrophobic region of the bilayer principally on the basis of hydrophobic interactions. The amino-terminal helix formed by the leader sequence is oriented with its amino terminus at the cytoplasmic side. The second helix is connected to the first through a short peptide loop on the periplasmic side.

Secondary structure calculations (data not shown) reveal that both the N-terminal hydrophobic region (residues 1 to 28) and the domain encompassing residues 34 to 54 have high alpha helix potentials. The latter region appears to be relatively polar, since it contains one Lys, one Arg, two Glu, two Thr and three Ser residues. However, based on free-energy calculations, Engleman and Steitz (8) have suggested that the energetic requirement of burying serines and threonines in a nonaqueous environment is not significant. Moreover, when acidic and basic amino acids occur three to four residues apart, a salt link can form between them which would greatly lower the energetic barrier to entry of these charged residues into a nonaqueous environment. Such salt bridges could form between Lys-44 and Glu-48 and between Glu-49 and Arg-53. It is therefore possible that the region encompassing residues 34 to 54 could form the second required alpha helix and that this helix would have a favorable free energy for partitioning into the lipid bilayer.

Based on the foregoing considerations, the amino-terminal region of PAK pilin (residues 1 to 54) is an excellent candidate for a helical hairpin. The positively charged six-residue leader, which is predicted to be located on the cytoplasmic side of the inner membrane, would presumably be cleaved in *P. aeruginosa* cells prior to pilus assembly. However, the present studies with *E. coli* suggest that leader cleavage is not obligatory for pilin transport to the inner membrane.

pBP001 contains two palindromic sequences distal to the pilin gene. The first is capable of forming a 12-nucleotide inverted repeat with a 4-nucleotide loop starting 143 nucleotides downstream of the stop codon of pilin. This structure would have a predicted free energy of -30.4 kilocalories (ca. -127 kJ) as predicted by the program of Queen and Korn (19). The end of the downstream stem consists of seven thymines, five of which may be involved in stem formation. The other potential hairpin region starts 152 nucleotides downstream of the stop codon, and has a predicted free energy of -24 kilocalories (ca. -100kJ).

mRNA stability is believed to be enhanced by repetitive extragenic palindromic sequences in *E. coli* and *Salmonella typhimurium* (23). These stable palindromes are thought to form hairpin loops in the mRNA, preventing RNA degradation (23). The palindromic regions from *P. aeruginosa* could stabilize the mRNA from degradation within *E. coli* in a manner similar to the repetitive extragenic palindromic sequence mechanism. This would explain the apparent high level of unprocessed pilin being produced and is analogous to the expression of the *B. nodosus* pilin gene in *E. coli* (2), which contains a 10 nucleotide inverted repeat followed by three thymines distal to its pilin gene. However, preliminary data suggests that these inverted repeats are not required for high levels of pilin expression within *E. coli* (unpublished results). Stem and loop structures followed by several thymines are also characteristic of [*rho*]-independent terminators (21). It is therefore possible that the region starting 142 residues downstream of the pilin stop codon also functions as a transcription termination signal.

Pilin genes from the virulent organisms *B. nodosus* (2), *N. gonorrhoeae* (16), *M. bovis* (13) and *P. aeruginosa* PAK (18) have all been cloned and shown to be expressed in *E. coli*. These pilin genes all share a very similar amino acid sequence within the first 28 amino acids of processed pilin. In addition, they all have a similar short (6- to 7-residue), positively charged leader sequence. Cloned pilin genes from *B. nodosus*, *N. gonorrhoeae*, and *P. aeruginosa* were all expressed efficiently in *E. coli* independent of vector promoter. In addition, *B. nodosus* pilin was found in the membrane fraction of *E. coli* (2). Since the leader sequences and hydrophobic regions are conserved in these diverse gram-negative organisms, they may follow similar transport pathways.

We have presented here a unique system in which a membrane protein from *P. aeruginosa* was shown to be expressed within *E. coli* without a vector promoter. Its promoter strength was assayed, and its cellular location examined. This information should facilitate studies of gram-negative protein transport and expression of virulence factors within *E. coli*.

FIGURE IV.1

(A) Autoradiograph of a 15% SDS-polyacrylamide gel containing ^{35}S-labeled minicells of pUC8 (lane 1) and pBP001 (lane 3). The bar (lane 2) represents the mobility of purified unlabeled *P. aeruginosa* PAK pilin. The arrows represent the mobility of size standards of (from the top) 43, 25.7, 18.4, 14.3, 12.3, 6.2 and 3 kilodaltons. (B) Immunoblot of unlabeled minicells from pUC8 (lane 1) and pBP001 (lane 2). Lane 3 contains 2 μg of purified PAK pilin.

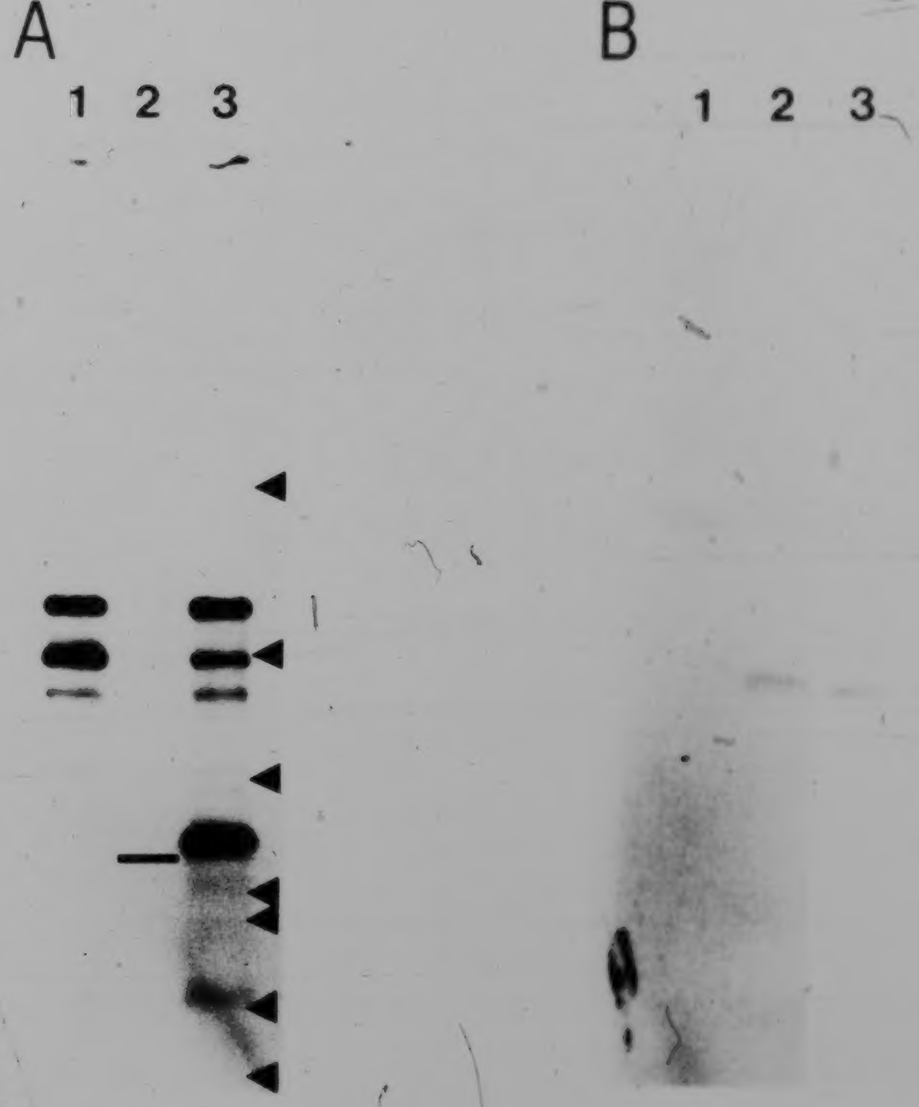

A
1 2 3
B
1 2 3

FIGURE IV.2

Autoradiograph of a 15% SDS-polyacrylamide gel containing cell fractions from ^{35}S-labeled minicells containing pBP001. For each fraction, 30,000 cpm was loaded. Lanes: 1, cytoplasm; 2, periplasm; 3, total membrane; 4, unfractionated minicells. The bar (lane 5) represents the mobility of PAK pilin. The arrows (lane 6, from the top) represent the mobility of the size standards described in Fig. 1.

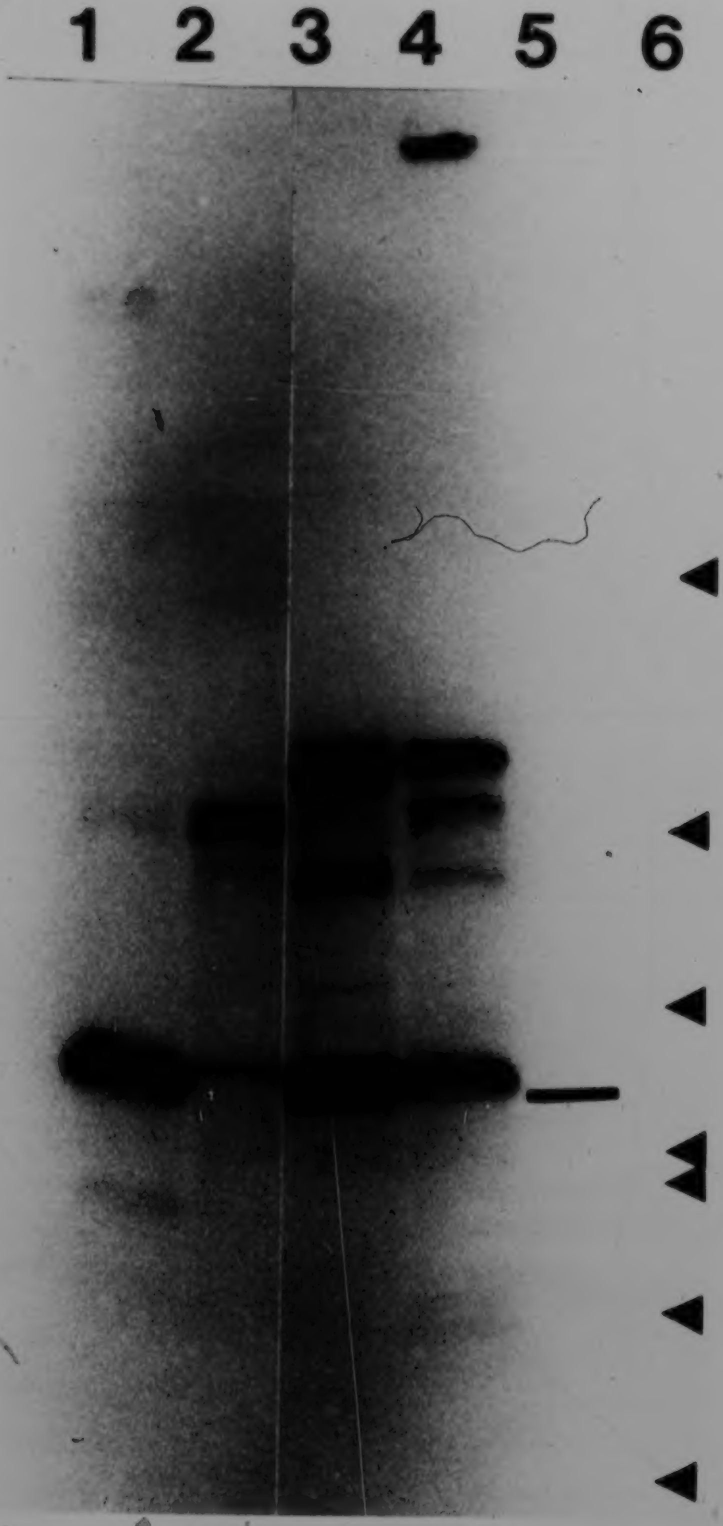
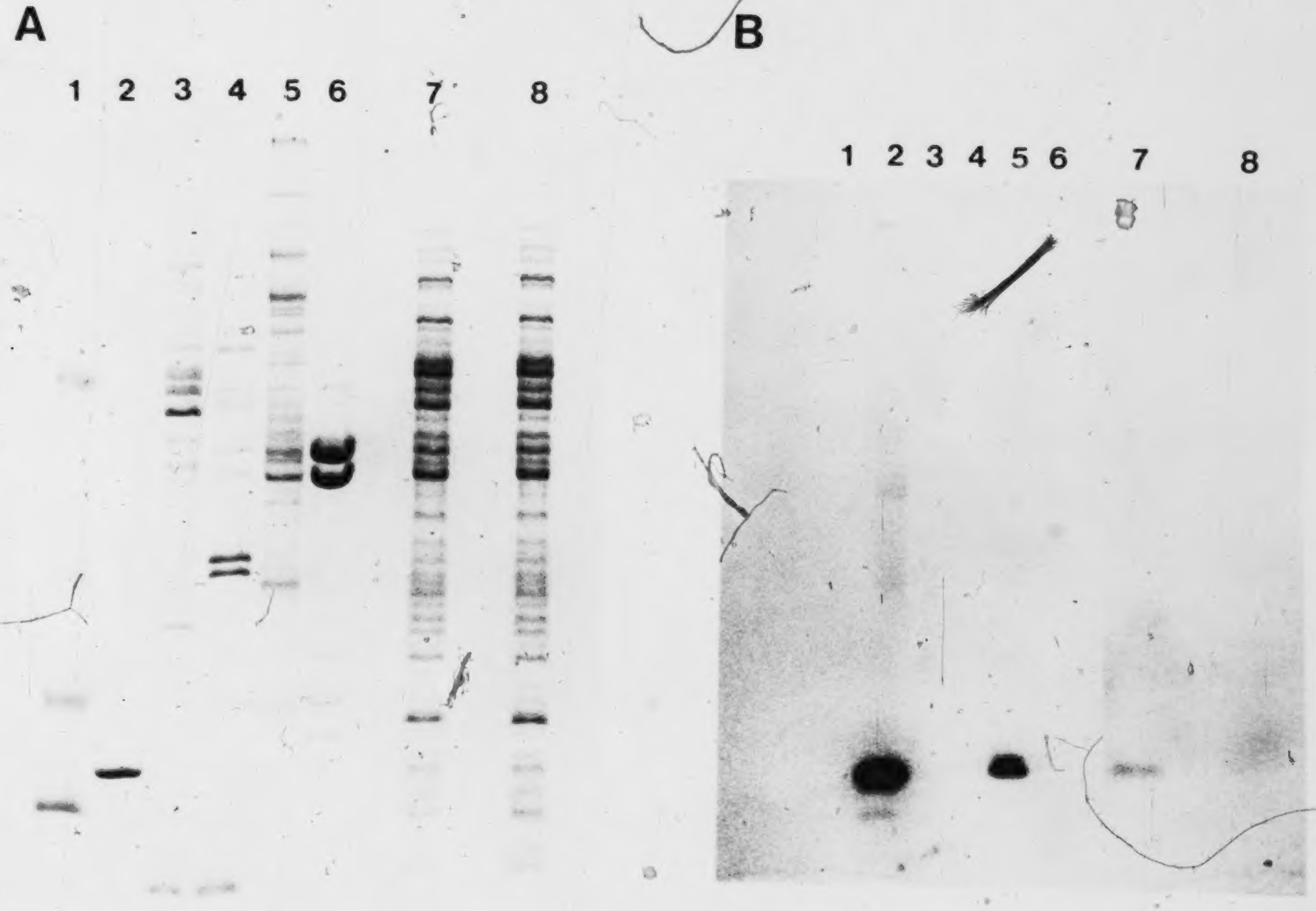

1
2
3
4
5
6

FIGURE IV.3

Subcellular localization of *P. aeruginosa* prepilin and pilin proteins in *E. coli* HB101 transformed with pBP001. Approximately equivalent amounts (on a weight basis) of the various cell fractions were electrophoresed on 15% SDS-polyacrylamide gels. (A) Coomassie blue-stained gel. Lanes: 1, molecular size standards (from the top) 43, 25.7, 18.4 and 14.3 kilodaltons; 2, 4 µg of PAK pilin; 3, cytoplasm; 4, periplasm; 5, inner membrane; 6, outer membrane; 7, unfractionated cells. Lane 7 contains 1/10 the number of cells used to obtain the fractions shown in lanes 3 to 6. Lane 8 contains unfractionated HB101 cells. (B) Immunoblot of the gel in panel A, containing identical samples as used for panel A except that 0.2 µg of PAK pilin was used. The anti-PAK pili serum used was preabsorbed against HB101.


A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8

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Chapter V

THE EXPRESSION OF *PSEUDOMONAS AERUGINOSA* PAK PILIN GENE MUTANTS IN *ESCHERICHIA COLI*[1]

A. INTRODUCTION

In prokaryotes, proteins destined for the periplasm or outer membrane are generally synthesized in a precursor form characterized by an extended amino terminus of 15 to 25 residues (known as a signal sequence; Michaelis and Beckwith, 1982). Unprocessed NMePhe pilins (prepilin) are unique in that, depending on the particular genera or strain of bacteria chosen, they have an N-terminal extension of 6 or 7 residues with 1 or 2 positive charges as determined from DNA sequencing (Elleman and Hoyne, 1984; Meyer *et al.*, 1984; Marrs *et al.*, 1985; Sastry *et al.*, 1985a). This short sequence is cleaved from prepilin prior to, or during, pilus assembly and the highly conserved N-terminal region of the NMePhe pilins may act as a signal sequence because of its similarity to conventional leader sequences. We mutated the *Pseudomonas aeruginosa* PAK pilin gene to delineate the regions of pilin necessary for stability and transport in *Escherichia coli*. Our results suggest that the C-terminal half of pilin interacts with the N-terminal region during pilin transport into

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the cytoplasmic membrane. As previously hypothesized (Finlay *et al.*, 1986), we present data which supports the theory that the first third of prepilin may conform to a "helical hairpin" structure within the cytoplasmic membrane. We also studied the transport of a peptide, having the N-terminal 36 amino acids of prepilin, reflecting the characteristics basic to a typical signal sequence. *In vivo* expression of this signal sequence-like peptide determined that it associated exclusively with the membrane fraction of *E. coli* which suggested a posttranslational mechanism for translocation of the peptide into the membrane.

B. MATERIALS and METHODS

1. Bacterial Strains and Plasmids.

The bacterial strains used were *E. coli* strains JM83 (Viera and Messing, 1982), K38 (Russel and Model, 1984) and HB101 (Boyer and Rolland-Dussoix, 1969). The plasmids pT7-4 and pGP1-2 (Tabor and Richardson, 1985) were kindly provided by Stan Tabor (Department of Biological Chemistry, Harvard Medical School, Boston, Mass. 02115).

2. Media.

Luria-Bertani (LB) medium was previously described (Maniatis *et al.*, 1982). Labeling media was minimal media (Miller, 1972) supplemented with 0.004% threonine, 0.004% leucine, and 1% Methionine Assay Powder. Antibiotic concentrations were 50 μg/ml for both ampicillin and kanamycin and 200 μg/ml for rifampin (stock solution 20 mg/ml in methanol).

3. Protein Sequencing.

Prepilin and the prepilin deletion mutants were transferred onto GF/C glass fiber paper using the acid transfer method (Aebersold *et al.*, 1986). The transfer occurred over a 12 h period in a buffer of 2% (vol/vol) acetic acid and 0.5% (vol/vol) Nonidet P-40. Approximately 700 pmole of protein was loaded onto a 15% SDS-PAGE (sodium dodecyl-sulfate-polyacrylamide gel electrophoresis) (Laemmli, 1970) and the efficiency of transfer to the GF/C paper was 10 to 20%. Automated Edman degradation of the electroblotted peptides was performed in a gas-liquid sequencer (Applied Biosystems Model 470A) (Hewick *et al.*, 1981) equipped with an on-line HPLC (Applied Biosystems Model 120A) for PTH-amino acid identification.

4. Oligonucleotide-Directed Mutagenesis.

The synthetic oligonucleotide 5' CTCGTTAGGAAGGC 3' was kindly provided by Mike Smith (Department of Biochemistry, University of British Colombia, Vancouver, BC, Canada V6T 1W5). The single-stranded vector procedure (Smith, 1985) was used to construct the site specific mutant coding for peptide 36.

5. Recombinant DNA Techniques.

DNA sequencing and recombinant DNA techniques were performed as previously described (Pasloske *et al.*, 1985).

6. Protein Overproduction and Labeling.

The procedure for labeling the truncated prepilin mutants with [^{35}S]methionine was as follows. *E. coli* K38 cells, transformed with both pGP1-2 and the pT7-4 plasmid with the desired insert, were grown in 2 ml of LB medium with kanamycin and ampicillin at 30°C. At an A_{590}=0.5, 0.2 ml of the cells were centrifuged, washed with labeling media and recentrifuged. The cells were resuspended in 1 ml of labeling medium and incubated with shaking at 30°C for 30 min. The temperature was raised to 42°C for 15 min. A 10 µl volume of rifampin was added and the cells were incubated 10 min at 42°C. Another 20 min incubation at 30°C was followed by the addition of 10 µCi of [^{35}S]methionine (Dupont, Lachine, Que., Canada). After 5 min at 30°C, the cells were centrifuged and subjected to SDS-PAGE. The 15% acrylamide gels were soaked in water for 30 min followed by 30 min in 1 M sodium salicylate, and then vacuum-dried before exposure to X-ray film.

To overproduce the prepilin mutants, K38 cells transformed with pGP1-2 and the pT7-4 recombinant plasmids were grown to an A_{590}=0.85 in 200 ml of LB in the presence of kanamycin and ampicillin at 30°C. The cells were incubated for 25 min at 42°C before adding 1 ml of rifampin. The cells were ready for fractionation after 2 h shaking at 37°C.

7. Immunoblotting.

The cell fractions were subjected to SDS-PAGE and the fractionated proteins were electrophoretically transferred to nitrocellulose sheets (Towbin *et al.*, 1979). The sheets were reacted with anti-PAK pili serum followed by incubation with ^{125}I-labeled protein A (Lachine, Que., Canada) (Watts *et al.*, 1983). Autoradiography of the nitrocellulose sheets was used to detect the proteins cross reacting with the antiserum.

8. Whole Cell Fractionation.

Fractionation of unlabeled whole cells was performed as previously described (Finlay *et al.*, 1986).

The fractionation of whole cells labeled with [^{35}S]methionine was similar to the minicell fractionation (Finlay *et al.*, 1986) except that during the lysozyme step, the cells were left on ice for 30 min instead of at 37°C for 1 h. Also, the periplasmic and cytoplasmic fractions were dried in a rotoevaporator instead of being precipitated with 10% TCA.

C. RESULTS

1. Construction of the Pilin Mutants.

In earlier work (Finlay *et al.*, 1986), it was proposed that the helical hairpin insertion model of Engelman and Steitz might be applicable to *P. aeruginosa* PAK pilin. In an attempt to verify this possibility, we constructed a clone coding for a peptide having just the first third of the prepilin sequence (which would theoretically form the hairpin) to ascertain whether this peptide could insert into the cytoplasmic membrane of *E. coli*. To engineer this peptide, we used recombinant DNA technology to manipulate pBP001, the chimera with the originally cloned

1.2-kilobase *Hin*dIII fragment encompassing the PAK pilin gene ligated into pUC8 (Pasloske *et al*., 1985). Initially, the *Hin*dIII fragment was shortened to the *Sal*I site at position 1123 to create pBP007 (Fig. 1). We then took advantage of the the *Hae*II fragment between positions 623 and 919 and deleted it from pBP007 which truncated the prepilin protein at Ala$_{50}$ and fused it to another nine amino acids at its C-terminus. This construct, called pBP021, coded for the first 56 residues of prepilin and nine amino acids derived from the vector, to form a fusion peptide of 65 amino acids and was thus named peptide 65 (Fig. 2). Also available were three *Sau*3A restriction sites grouped at positions 485, 497, and 509. Deletions within pBP021 were created by removing a *Sau*3A fragment between positions 485 and 497 (pBP023) and by deleting the two *Sau*3A fragments between positions 485 and 509 (pBP022). These constructs coded for peptide 65 with amino acids 4 to 7 or 4 to 11 deleted from the conserved N terminus and they were designated peptides 65Δ4 and 65Δ8 respectively (Fig. 2).

To complete this series of mutants, the *Hae*II fragment deleted from pBP007 was ligated back into the single *Hae*II sites of pBP022 and pBP023, giving rise to pBP012 and pBP013, respectively. These insertions regenerated complete pilin genes except that they had either a 4 or an 8 amino acid N-terminal deletion such that the translated products were named prepilinΔ4 and prepilinΔ8, respectively (Fig. 2).

Peptide 36 (Fig. 2) was used to determine whether the hydrophobic, N-terminal region of prepilin alone was sufficient for transport into an *E. coli* membrane. Using site specific mutagenesis, the clone pBP026 was created in which the TCG codon for Ser_{31} was replaced with a TAG stop codon so that the C-terminal residue became Arg_{30}, resulting in a mutant with only the first 36 amino acids of unprocessed pilin.

2. Pilin Expression Using the T7 Promoter/Polymerase System.

A T7 promoter/polymerase system was used to achieve a high level of expression of the above clones (Tabor and Richardson, 1985). This system involves the use of two plasmids transformed into the same *E. coli* cell, as described in *Materials and Methods*. The plasmid pGP1-2 encodes the temperature sensitive lambda repressor (cI857) which regulates the expression of the T7 polymerase encoded within the same plasmid. The other plasmid, pT7-4, has a multiple cloning site downstream of a T7 promoter into which the gene of choice can be cloned. At 30°C, the lambda repressor inhibits the transcription of the T7 polymerase. At 42°C, the repressor becomes inactivated allowing the transcription of the T7 polymerase which then transcribes the DNA downstream of the T7 promoter in the pT7-4 plasmid.

Specific labeling of the protein expressed by the pT7-4 clone was possible by the addition of rifampin before adding the $[^{35}S]$methionine to the cells. This drug inactivates the host RNA polymerase but does not affect the T7 polymerase so that only genes under the T7 promoter are transcribed to generate mRNA which are translated with the incorporation of $[^{35}S]$methionine.

Table 1 gives the names of the pT7-4 chimeras, derived from the original pUC8 clones, and their peptide products. The pUC8 chimeras were digested with *Hin*dIII and *Eco*RI. The cloned fragments were isolated and ligated into the *Hin*dIII and *Eco*RI sites of pT7-4.

3. Localization of the Pilin Mutants.

Using the T7 system of expression, pilin and the other six pilin mutants were overproduced in *E. coli* and then the cells were fractionated. Each fraction was subjected to SDS-PAGE and stained with Coomassie blue. It was not possible to detect the expression of any of the truncated pilin mutants which would have been produced by pBP031, pBP032, pBP033, and pBP036 in any of the cellular fractions. However, among the cells expressing prepilin, prepilinΔ4, and prepilinΔ8, it was observed that these proteins were localized primarily to the cytoplasmic membrane and each of these proteins had mobilities (molecular weights were approximately 15,000) correlating to their unprocessed forms (Fig. 3). Immunoblots of these gels with anti-PAK pili serum were positive for these same proteins; however, there was no cross-reactivity of the antiserum with the truncated pilins which was expected since the immunodominant region of pilin was missing (Sastry *et al.*, 1985b). The three different pilin proteins were produced in sufficient quantities to allow their N termini to be sequenced by using an electroblotting technique (Aebersold *et al.*, 1986). After the cytoplasmic membrane was fractionated by SDS-PAGE, the pilins were transferred onto GF/C glass fiber discs. Gas phase sequencing of these discs confirmed that all three proteins were unprocessed, the N-terminal residue being a free methionine, and that the two mutants had the predicted deletions of 4 or 8 amino acids.

Although the three pilins localized primarily to the inner membrane, lesser quantities of the protein were also observed in the outer membrane and the cytoplasm by Coomassie blue staining (data not shown).

To prove that the fractions containing the cytoplasm and outer membrane were not contaminated with the pilin due to overproduction of the proteins, *E. coli* HB101 was transformed with pT7-4, pBP009, pBP014 or pBP015 in an effort to lower the levels of transcription of the pilin genes. In this case, transcription of the pilin genes would occur from the beta-lactamase promoter and the fortuitous *E. coli* promoter just upstream of the pilin gene as recently proposed (Finlay *et al.*, 1986; Johnson *et al.*, 1986). After fractionation, the pilins were not visible in the cytoplasmic membrane by Coomassie blue staining as they were when they were overproduced by the T7 polymerase system (Fig. 4A). Detection of the pilins was only possible by immunoblotting with anti-PAK pili serum which revealed that the three different pilins were localized primarily to the cytoplasmic membrane and they were also weakly detected in the cytoplasmic and the outer membrane fractions (Fig. 4B). This distribution to other cellular fractions has been observed with other *Pseudomonas* pilin transport studies in *E. coli* involving alkaline phosphatase fusions (Strom and Lory, 1987). It is worth noting that the cytoplasmic membrane fraction was slighty contaminated with the outer membrane fraction as evidenced by the OMP proteins at about 35,000 molecular weight. However, the immunoblot showed more intense pilin bands in the cytoplasmic membrane fraction than in the outer membrane fraction even though greater amounts of the outer membrane fractions had been loaded, indicating that the majority of the pilin was in the cytoplasmic membrane fraction. It is interesting that the wild type pilin appeared in its unprocessed and degraded form at molecular weight 15,000 while prepilinΔ4 and prepilinΔ8 migrated according to their unprocessed forms only, suggesting that the N-terminal deletions inhibited pilin degradation.

4. Detecting the Truncated Pilin Mutants.

Since the truncated pilins, expressed using the T7 system, could not be observed by staining with Coomassie blue, we resorted to radiolabeled isotopes for detection. When the cells transformed with the truncated pilin genes, were labeled with [^{35}S]methionine using the T7 polymerase system, and the whole cells were subjected to SDS-PAGE, the autoradiograph revealed that all peptides were detectable except for peptide 65 8 (Fig. 5). These peptides had molecular weights of 4,000 to 7,500. Peptide 65Δ4 did not appear as a full length product but as the lower molecular weight derivative, peptide 65Δ4$_a$ (see below, parts 5 and 6). The lack of appearance of peptide 65Δ8 was not due to weak expression at the level of transcription, since the beta-lactamase gene which was downstream of the peptide 65Δ8 gene, was expressed from the same mRNA transcript. Since the peptide 65Δ8 mRNA had the same 5' coding region as the prepilinΔ8 mRNA, and the latter was well expressed, translation was probably not affected. Therefore, the absence of the peptide 65Δ8 was probably due to proteolytic degradation.

5. The Localization and the Effects of Phenethyl Alcohol on the Truncated Pilins.

When the cells expressing the truncated pilins were labeled and fractionated, peptides 36, 65, and 65Δ4 were each found to be localized exclusively to the membrane fraction (Fig. 6). Besides the peptide 65 product, there were two other lower molecular weight peptides. To increase the stability of the nascently translated peptides, phenethyl alcohol was used during the labeling experiment to inhibit membrane associated peptidases (Halegoua and Inouye, 1979). This treatment resulted in an increase in the amounts of the nascent peptides for peptides 65 and 65Δ4 but peptide 36 was not affected by phenethyl alcohol (Fig. 7).

6. Pulse-Chase Experiment of the Truncated Pilins.

To assess the rates of proteolysis of the truncated pilins, a pulse-chase experiment was performed. The autoradiograph in Fig. 8 shows that peptide 36 was not degraded to detectable fragments over the 30 min period although it did gradually decrease in amount. The other two peptides were degraded at different rates to smaller fragments. Peptide 65 degraded to two smaller fragments. At 10 s, the amount of nascent peptide 65 was lower than the more stable degradation product (peptide 65_a). As peptide 65_a disappeared over the time course, another degradation product (peptide 65_b) appeared at about 15 min. Peptide 65Δ4 had only one stable product.

The nascent peptide 65Δ4 was barely detectable at 10 s while the single degradation product (peptide 65Δ4_a) was predominantly visible over the full time course. A second degradation product (peptide 65Δ4_b) did show up with much longer exposures and it migrated slighty faster than peptide 65_b (data not shown).

D. DISCUSSION

The N-terminal sequence of *P. aeruginosa* PAK unprocessed pilin has strong similarities to the typical signal sequences required by proteins destined for the periplasm or the outer membrane of gram-negative bacteria. prepilin has a positively charged N-terminus followed by a long hydrophobic stretch, qualities which are characteristic of signal sequences (Michaelis and Beckwith, 1982). The difference between the usual signal sequence and the N terminus of prepilin lies in the site of cleavage. Signal sequences have a polar C-terminal region after the stretch of hydrophobic amino acids which marks the site of cleavage (von Heijne, 1985). However, processing after this hydrophobic stretch has not been observed in *Pseudomonas* pilin even though an acceptable cleavage site has been identified between residues Ala_{20} and Ile_{21} of the mature pilin (Strom and Lory, 1987). prepilin is unique in that it lacks such a region which is recognized by the signal peptidase. Instead, processing occurs just after the the positively charged N-terminus and the mature pilin subunit maintains its N-terminal hydrophobic sequence. These similarities and differences led us to investigate the insertion properties of the unusual N-terminal sequence of prepilin by constructing mutants of pilin and studying their expression in *E. coli* to serve as a simple model system for transport of this *P. aeruginosa* protein.

The expression of *P. aeruginosa* PAK pilin was examined in *E. coli* using a T7 expression system and as demonstrated before (Finlay *et al.*, 1986), the pilin was localized primarily to the cytoplasmic membrane. The deletion of amino acids 4 to 7 or amino acids 4 to 11 from pilin did not interfere with the expression or transport of pilin to the cytoplasmic membrane. Secondary structure predictions (Chou and Fasman, 1978) spanning the regions in which the deletions were made, reveal strong increases in beta sheet potential corresponding with an obvious drop in helix potential (data not shown). These predictions lend strength to other proposals (Rosenblatt *et al.*, 1980; Steiner *et al.*, 1980; Briggs *et al.*, 1986) suggesting that a beta sheet conformation of the signal sequence is required for the transport of some proteins during the early stages of insertion into the membrane. In these models, transport would involve the signal sequence undergoing a transition from beta sheet to a helical structure as it partitioned into the membrane. Therefore, the secondary structure of the conserved N-terminal region of the NMePhe pili could be dependent on its environment since it has strong potential for both conformations (Sastry *et al.*, 1985a). An aqueous surrounding might induce a beta sheet conformation while insertion into a lipid bilayer might produce an alpha helical structure as previously demonstrated using physical techniques with the synthetic peptides for the signal sequences of preproparathyroid hormone and the LamB protein (Rosenblatt *et al.*, 1980; Briggs *et al.*, 1986). As well, a synthetic peptide for the M13 signal sequence preferred a helical conformation in a hydrophobic environment while in an aqueous solution, there was 70% random coil (Shinnar and Kaiser, 1984).

Previously, in addition to unprocessed pilin, a pilin product was detected in *E. coli* HB101 which had the same mobility as mature pilin in this study and in previous reports (Finlay *et al.*, 1986). This product may be the result of degradation of the prepilin at the N-terminus or C-terminus or both termini. The molecular weights of the prepilinΔ4 and prepilinΔ8 products correspond to the unprocessed forms alone while the wild type pilin was present as two polypeptides, one unprocessed and one corresponding to the processed molecular weight form. Thus, the deletions at the N terminus affected the degradation of the deletion mutant proteins, suggesting that the cleavage site of the wild type prepilin was within the N terminus. Unfortunately, insufficient material prevented sequencing the N terminus of the lower molecular weight form of the wild-type pilin. However, protein sequencing of the N termini of overproduced prepilin, prepilinΔ4 and prepilinΔ8, produced by the T7 polymerase system, verified that these products had intact leader peptides with free methionines as their N-terminal amino acids. The processed form of pilin was not detected possibly because the large amounts of pilin overloaded the capabilities of the *E. coli* peptidase which was weakly active in the *E. coli* HB101 system.

The truncated pilin mutants were constructed to ascertain the minimum length of an N-terminal signal-like sequence of pilin required for stable expression and membrane insertion in *E. coli*. Peptides 36, 65, and 65Δ4 but not 65Δ8 were detected within whole cell fractions by labeling with [^{35}S]methionine using the T7 system of expression. It is interesting that the expression of prepilinΔ8 was observed in *E. coli* while its truncated version (peptide 65Δ8) was not detectable in autoradiographs. This result suggests that the C-terminal region of pilin interacts with the N terminus to stabilize the deleterious effects of an 8 amino acid deletion. Perhaps peptide 65Δ8 can not assume the correct structure for transport into the membrane and is degraded rapidly while prepilinΔ8 is more stable because the C-terminal domain stabilizes the N terminus into a transport competent conformation. Cover *et al.* (1987) have shown that a second C-terminal mutation in the maltose-binding protein corrected a mutation in the leader sequence which had initally made it transport deficient. Thus, in cases where a protein is inserted posttranslationally, the C-terminal portion may have an active role in its transport.

Fractionation of the cells producing the truncated peptides demonstrated that peptides 36, 65, and 65Δ4 were localized exclusively to the membrane fraction. These data suggest that the first 36 amino acids of prepilin were sufficient for partitioning into the membrane, although it is also possible that we observed a non-specific association between peptide 36 and the cytoplasmic membrane. Since approximately 25 to 35 amino acids are embedded in the large ribosomal subunit during translation, the synthesis of peptide 36 would essentially be complete before it dissociated from the ribosome. This is reminiscent of the 70 residue precursor of the bee venom mellitin translocating across microsomal membranes (Wickner and Lodish, 1985). The transport of other proteins in *E. coli*, such as M13 procoat protein, beta-lactamase, and ribose-binding protein, has also been shown to be entirely posttranslational (Goodman *et al.*, 1981; Koshland and Botstein, 1982; Randall, 1983). Therefore, peptide 36 might partition into the membrane posttranslationally as might the pilin subunit itself. A posttranslational insertion mechanism would explain how the C-terminal half of prepilin$\Delta 8$ is able to influence the structure of the N-terminus for effective transport into the cytoplasmic membrane.

Recent work has suggested that the N-terminal region of pilin essential for membrane translocation lies between Met_{-6} and Ile_4 to Thr_{45} using alkaline phosphatase fusions (Strom and Lory, 1987). The work described here involving peptide 36 shortens this zone 15 residues from Met_{-6} to between Ile_4 and Arg_{30}.

The data gathered from the phenethyl alcohol and pulse-chase experiments disclosed information regarding the stabilities of the peptide mutants. The results showed that peptide 36 was a relatively stable product when expressed in *E. coli* whereas membrane peptidases produced peptides 65_a, 65_b, and $65\Delta 4_a$ (Fig. 8). To understand the nature of these degradation products, a review of the methionine positions is useful (Fig. 2). Peptide 65 could be labeled at three positions (Met_{-6}, Met_7, and Met_{58}) with [^{35}S]methionine while the deletion within peptide $65\Delta 4$

removed Met_7 which allows for labeling at both ends of the peptide. Assuming that peptides 65 and 65Δ4 were hydrolyzed at equivalent peptide bonds, the third degradation product of peptide 65Δ4 (peptide $65\Delta4_b$), which was visible after very long exposures could not have been detected if degradation had occurred at both ends of the peptide, in effect removing the two methionines at the N and C termini. Therefore, degradation could only have occurred at one end of the peptides. Degradation probably occurred at the C terminus since all the detectable products of peptide 65Δ4 had slightly faster mobilities than the products of peptide 65, due to the 4 amino acid deletion. If degradation had occurred at the N terminus at equivalent sites, we would have expected peptides 65_b and $65\Delta4_b$ to be the same molecular weight.

The degradation pattern produced in the pulse-chase experiment suggests a plausible model for the membrane-associated structure of the first 50 residues of prepilin, as depicted in Fig. 9. If peptides 65 and 65Δ4 both assume a hairpin conformation in the cytoplasmic membrane such that the N and C termini face the cytoplasm and the predicted beta-bend involving residues Arg_{30} to Gly_{33} (Sastry *et al.*, 1985a) is in the periplasmic space, then the degradation products derived from peptides 65 and 65Δ4 are understandable. This model predicts two sites of hydrolysis; one extremely labile site at the C-terminal end of the peptides (site C), and the second site immediately following the beta bend in the periplasmic space (site P). Following translocation into the membrane, cleavage of peptide 65 at site C would happen quickly giving rise to peptide 65_a followed by the much slower proteolysis at site P producing peptide 65_b. Peptide 65_b migrated slightly faster than peptide 36 which ends at Arg_{30}, suggesting that site P is located before Arg_{30}. The *E. coli* signal peptidase may be responsible for the periplasmic cleavage at site P. A site between Val_{28} and Ala_{29} best conforms to the consensus sequence of a signal peptidase cleavage site (von Heijne, 1984).

The rates of formation of the degradation products of peptides 65 and 65Δ4, indicate that peptide $65\Delta4_a$ was produced more rapidly than peptide 65_a while peptide 65_b was produced quickly in comparison to peptide $65\Delta4_b$ which was barely detectable only after long exposures (Fig. 8). These considerations suggest that there may be two membrane peptidases acting at the two cleavage sites referred to above; one facing the cytoplasm (at site C) and the other facing the periplasmic space (at site P). The putative peptidase located at site C appeared to cleave peptide 65 relatively quickly, however, the deletion of 4 amino acids increased its efficiency of hydrolysis. This same 4 amino acid deletion altered site P into an unrecognizable substrate for the putative periplasmic membrane peptidase, thus lowering the rate of hydrolysis of peptide $65\Delta4_a$ to almost zero and making it very stable.

The strongly conserved homology of the first 30 amino acids of the NMePhe pilins suggests an important and precise function for this sequence. The N-terminus may be fulfilling a dual role having the responsibility of partitioning the pilin subunit into the membrane and being involved in subunit-subunit interactions within the assembled pilus. Ionization studies of Tyr_{24} and Tyr_{27} during the pH titration of PAK pili, before and after dissociation into dimers by octyl-glucoside, revealed that this hydrophobic region of pilin was involved in subunit-subunit interactions (Watts *et al.*, 1983).

Thus the N-terminal region has a structural role in the pilus and it can also partition into the membrane of *E. coli*, in a manner that is similar to that of a signal sequences. Because it has evolved towards fulfilment these two functions, the sequence of the N-terminal region must be stringently maintained or a single amino acid change could destroy either of its proposed roles, ultimately preventing pilus assembly.

We have shown that the deletion of 4 or 8 residues of the N terminus of pilin does not affect its apparent translocation into the membrane of *E. coli*. As well, only the first 36 amino acids of prepilin were required for membrane insertion, probably using a posttranslational mechanism. Studies with these mutants are now being performed in *P. aeruginosa* to ascertain the effect of these deletions on transport and processing. These experiments will be of value in elucidating some of the basic aspects of the transport of pilin in *P. aeruginosa*.

FIGURE V.1

Construction of the mutant pilin genes. Refer to the *Results* section for a more detailed explanation. Each of the constructs were originally cloned into the vector pUC8. The inserts were then ligated into the *Hin*dIII and *Eco*RI sites of pT7-4 for the expression of the mutant pilin genes using T7 polymerase. The bold arrow beneath each clone indicates the open reading frame of the particular pilin mutant and the name of the translated product. The abbreviations for the restriction enzymes are: *Hin*dIII (III), *Sal*I (SI), and *Hae*II (II). In this figure, propilin is equivalent to prepilin.

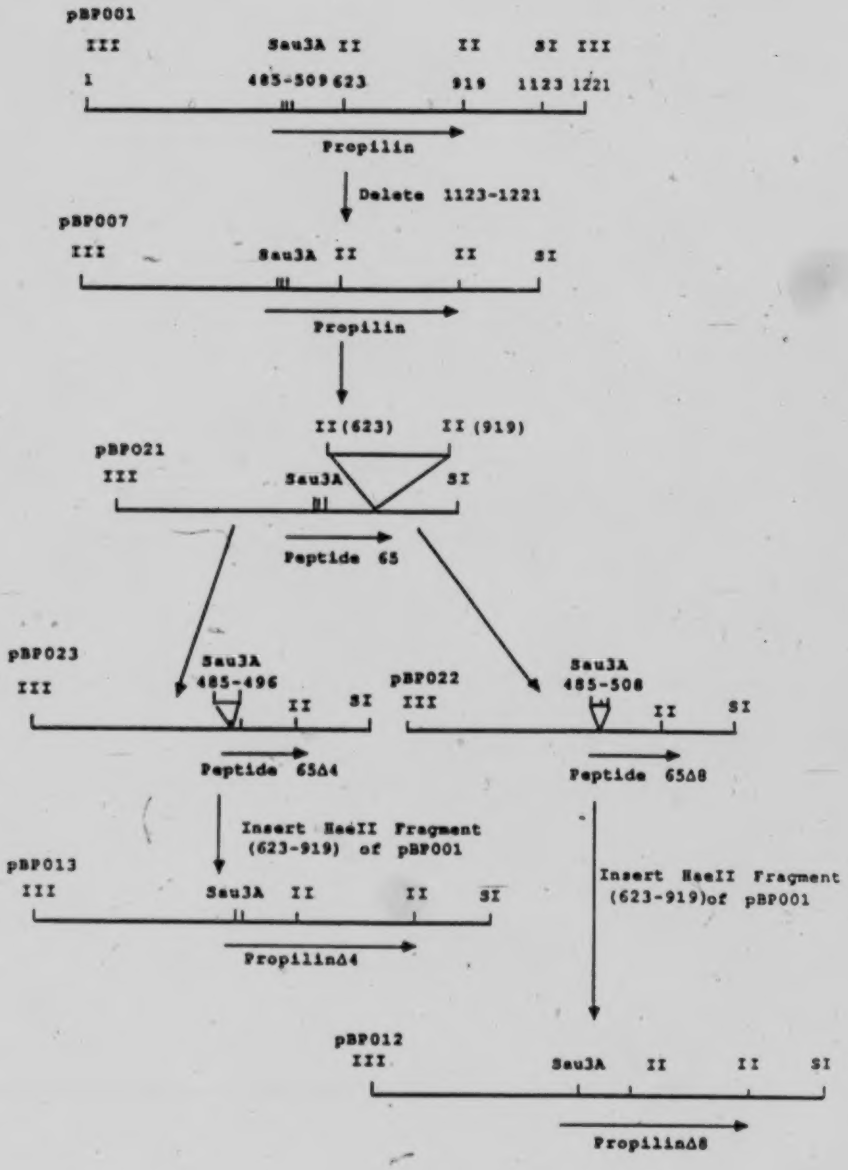
pBP001
III
Sau3A
II
II
SI
III
1
485-509
623
919
1123
1221
Propilin
Delete 1123-1221
pBP007
III
Sau3A
II
II
SI
Propilin
II(623)
II(919)
pBP021
III
Sau3A
SI
Peptide 65
pBP023
III
Sau3A
485-496
II
SI
Peptide 65Δ4
pBP022
III
Sau3A
485-508
II
SI
Peptide 65Δ8
Insert HaeII Fragment
(623-919) of pBP001
pBP013
III
Sau3A
II
II
SI
PropilinΔ4
Insert HaeII Fragment
(623-919)of pBP001
pBP012
III
Sau3A
II
II
SI
PropilinΔ8

FIGURE V.2

Amino acid sequences of the pilin mutants. The PAKwt pilin sequence is shown along the top line to position +59, which is then followed by periods representing the continuation of the sequence to residue +144 (Pasloske *et al.*, 1985). The arrow indicates the site at which processing occurs to produce mature pilin. At position +31 the serine residue is underlined, indicating the position of the stop codon created by site-specific mutagenesis to produce peptide 36. The slashed line (\) indicates where the fusion peptide begins for the three truncated pilin mutants created by the deletion of the *Hae*II fragment and the stars (***) at position +60 identify the stop codon. The dashes (---) show the deletion of 4 or 8 amino acids for prepilinΔ4 and peptide 65Δ4 or prepilinΔ8 and peptide 65Δ8 respectively.

```
         -5             +1                         +10
PAKwt MetLysAlaGlnLysGlyPheThrLeuIleGluLeuMetIleValValAlaIleIleGly
PAKΔ4 MetLysAlaGlnLysGlyPheThrLeu------------IleValValAlaIleIleGly
PAKΔ8 MetLysAlaGlnLysGlyPheThrLeu------------------------IleIleGly
                        ↑
                     +20                           +30
      IleLeuAlaAlaIleAlaIleProGlnTyrGlnAsnTyrValAlaArgSerGluGlyAla

                     +40                           +50
      SerAlaLeuAlaSerValAsnProLeuLysThrThrValGluGluAlaLeuSerArgGly
                                                     \LeuAspGlyVal

                  +59
      TrpSerValLysSer.........
      GluLeuSerMetIle***
```

FIGURE V.3

The cytoplasmic membrane fractions of the cells producing pilin and the pilin deletion mutants. Prepilin, prepilinΔ4, and prepilinΔ8 were expressed in *E. coli* K38 using the T7 polymerase system. After overproducing these proteins, the cells were fractionated and the cytoplasmic membrane fractions were subjected to SDS-PAGE and the gel stained with Coomassie blue. The size of the molecular weight standards ($x10^{-3}$) are printed along the left-hand side of the gel. In this figure, propilin is equivalent to prepilin.

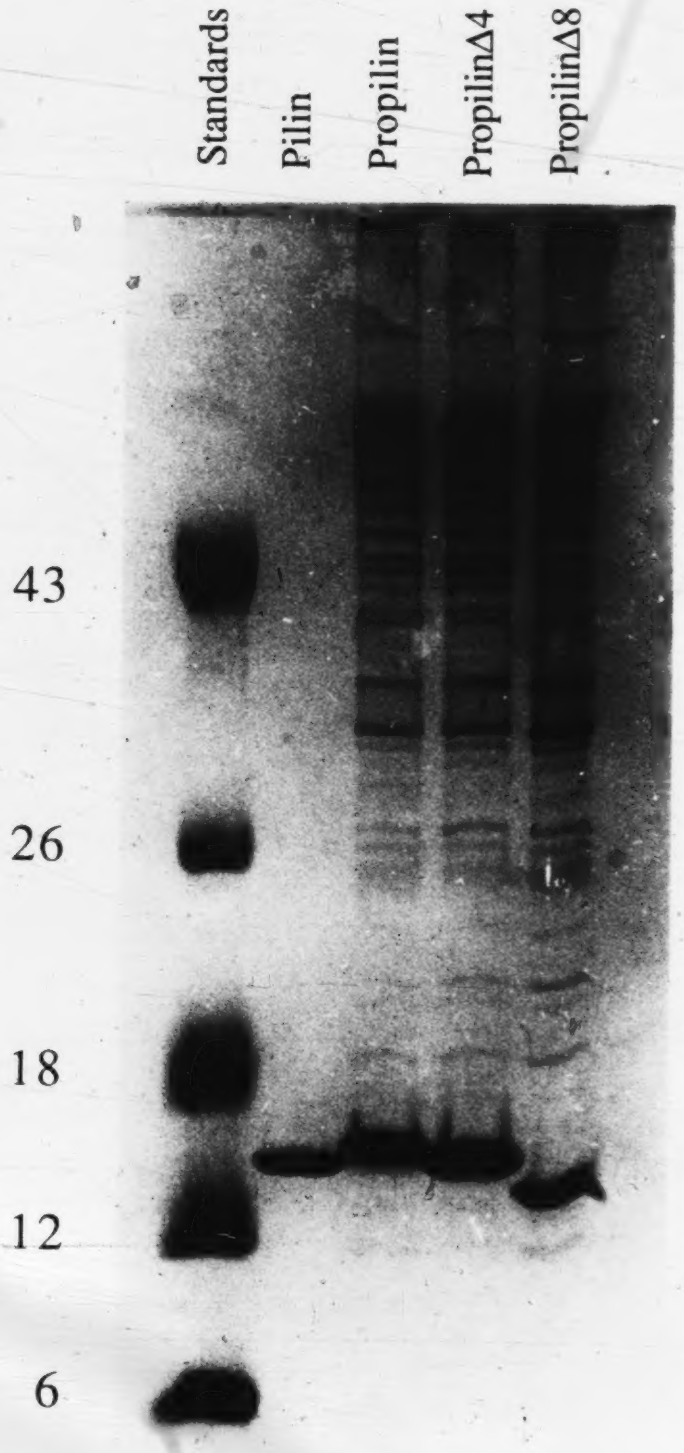
Standards
Pilin
Propilin
PropilinΔ4
PropilinΔ8
43
26
18
12
6

FIGURE V.4

Fractionation and immunoblot of the mutant pilins expressed in *E. coli* HB101. The vector pT7-4 and the pT7-4 chimeras expressing prepilin, prepilinΔ8, and prepilinΔ4 (lanes 1, 2, 3, and 4 respectively) were transformed into *E. coli* HB101. After fractionating these transformants, approximately equal amounts (on a weight basis) of the cell fractions, except for lower amounts of the cytoplasmic membrane fractions, were electrophoresed on 15% SDS-PAGE. A. Coomassie blue stained gel with 4 μg of PAK pilin loaded as a standard. B. The immunoblot of the identical gel in panel A using anti-PAK pili serum. An equivalent of 0.2 μg of PAK pilin was loaded as the standard. The size of the molecular weight ($x10^{-3}$) standards are printed on the left-hand side of the figure.

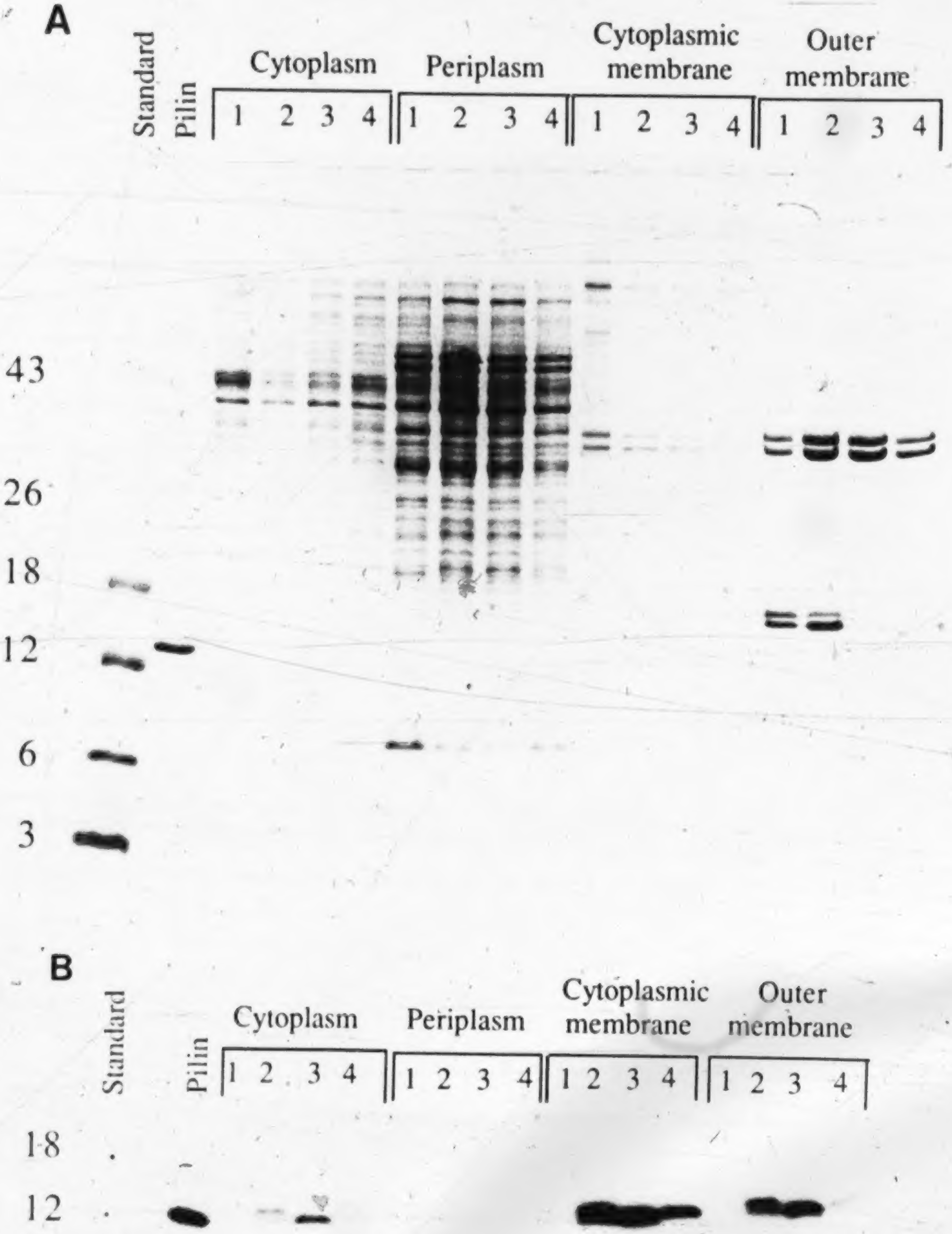
A
Standard
Pilin
Cytoplasm
1 2 3 4
Periplasm
1 2 3 4
Cytoplasmic membrane
1 2 3 4
Outer membrane
1 2 3 4
43
26
18
12
6
3
B
Standard
Pilin
Cytoplasm
1 2 3 4
Periplasm
1 2 3 4
Cytoplasmic membrane
1 2 3 4
Outer membrane
1 2 3 4
18
12

FIGURE V.5

Labeling of the whole cells transformed with the chimeras coding for the truncated pilin mutants. *E. coli* K38 cells transformed with the chimeras coding for the truncated pilin mutants were labeled with [^{35}S]methionine using the procedure as described in *Materials and Methods*. Whole cell samples containing equal counts for each mutant were subjected to SDS-PAGE. The size of the molecular weight standards ($\times 10^{-3}$) are printed on the left-hand side of the figure. The positions of the full length labeled peptides (peptides 65 and 36) and the degraded derivatives of peptides 65 and 65Δ4 (peptides 65_a and $65\Delta 4_a$, respectively) are indicated on the right-hand side of the panel.

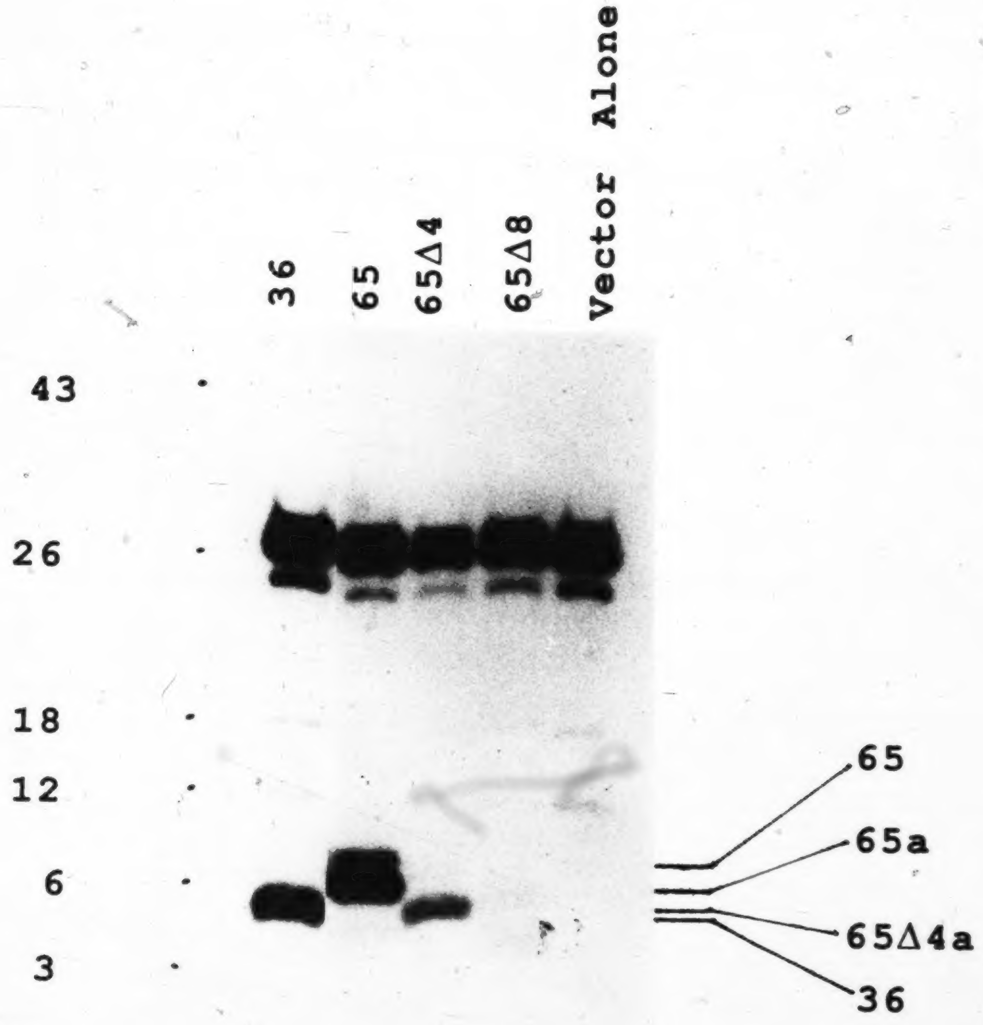
36
65
65Δ4
65Δ8
Vector Alone
43
26
18
12
6
3
65
65a
65Δ4a
36

FIGURE V.6

Autoradiogram of the fractionation of the cells expressing the truncated pilins. *E. coli* K38 cells producing the the truncated pilins were labeled and then fractionated. Equal counts were loaded in each lane of the protein gel. W, C, P, and M refer to the whole cell, cytoplasmic, periplasmic and membrane fractions, respectively. The positions of the labeled peptides and their derivatives are indicated along the right-hand side of the panel. The derivatives are signified with the subscript "a" or "b".

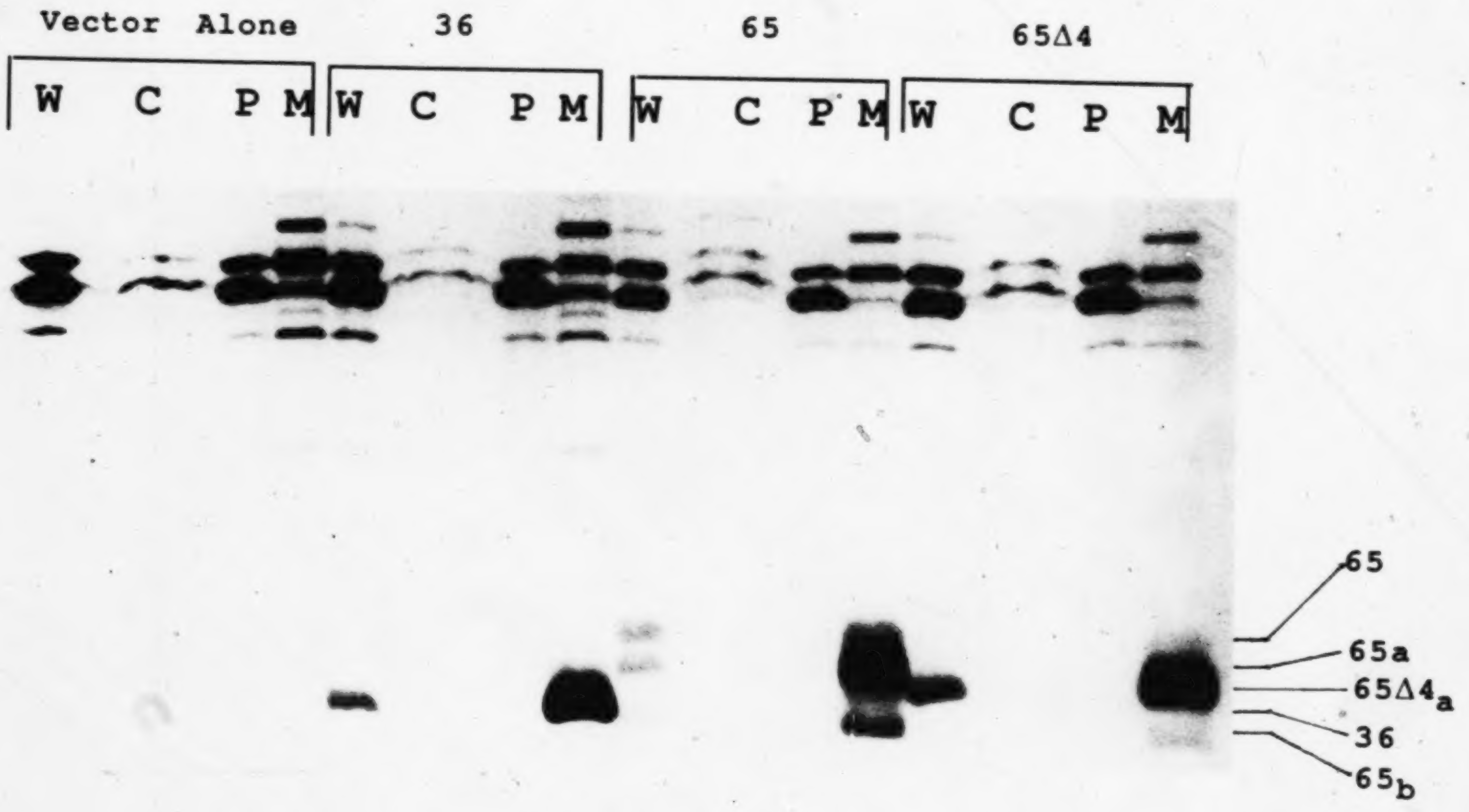
Vector Alone
36
65
65Δ4
W
C
P
M
W
C
P
M
W
C
P
M
W
C
P
M
65
65a
65Δ4a
36
65b

FIGURE V.7

Detecting the nascent truncated pilins. The *E. coli* K38 cells expressing the truncated pilins were assayed for the nascently translated peptides by inhibiting the membrane peptidases with the addition of 2 µl of phenethyl alcohol concommitant with the addition of the [^{35}S]methionine. The + and - signs refer to the addition of the phenethyl alcohol. The positions of the labeled peptides and their derivatives are indicated on the right-hand side of the panel. The derivatives are signified by the subscript "a".

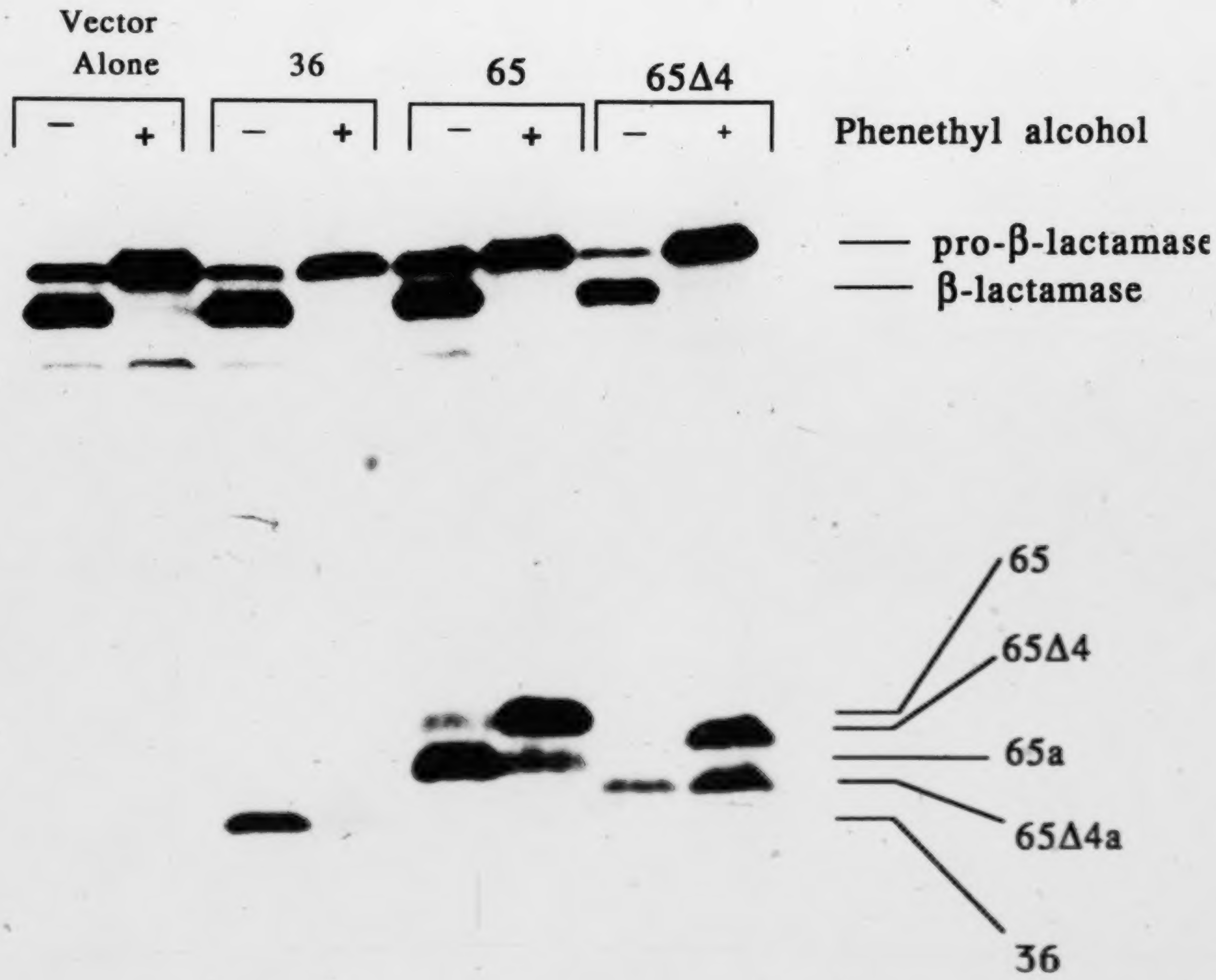
Vector
Alone
36
65
65Δ4
– +
– +
– +
– +
Phenethyl alcohol
pro-β-lactamase
β-lactamase
65
65Δ4
65a
65Δ4a
36

FIGURE V.8

Autoradiogram of a pulse-chase labeling of the truncated pilins. Cells expressing peptides 36, 65 and 65Δ4 were labeled with [^{35}S]methionine. After 1 min, 100 µl of 1% (wt/vol) unlabeled methionine was added, samples were taken at 10 s, 5 min, 15 min, and 30 min and the whole cell fractions were subjected to 15% SDS-PAGE. The positions of the labeled peptides and their derivatives are indicated along both sides of the panel (see *Results*, part 6).

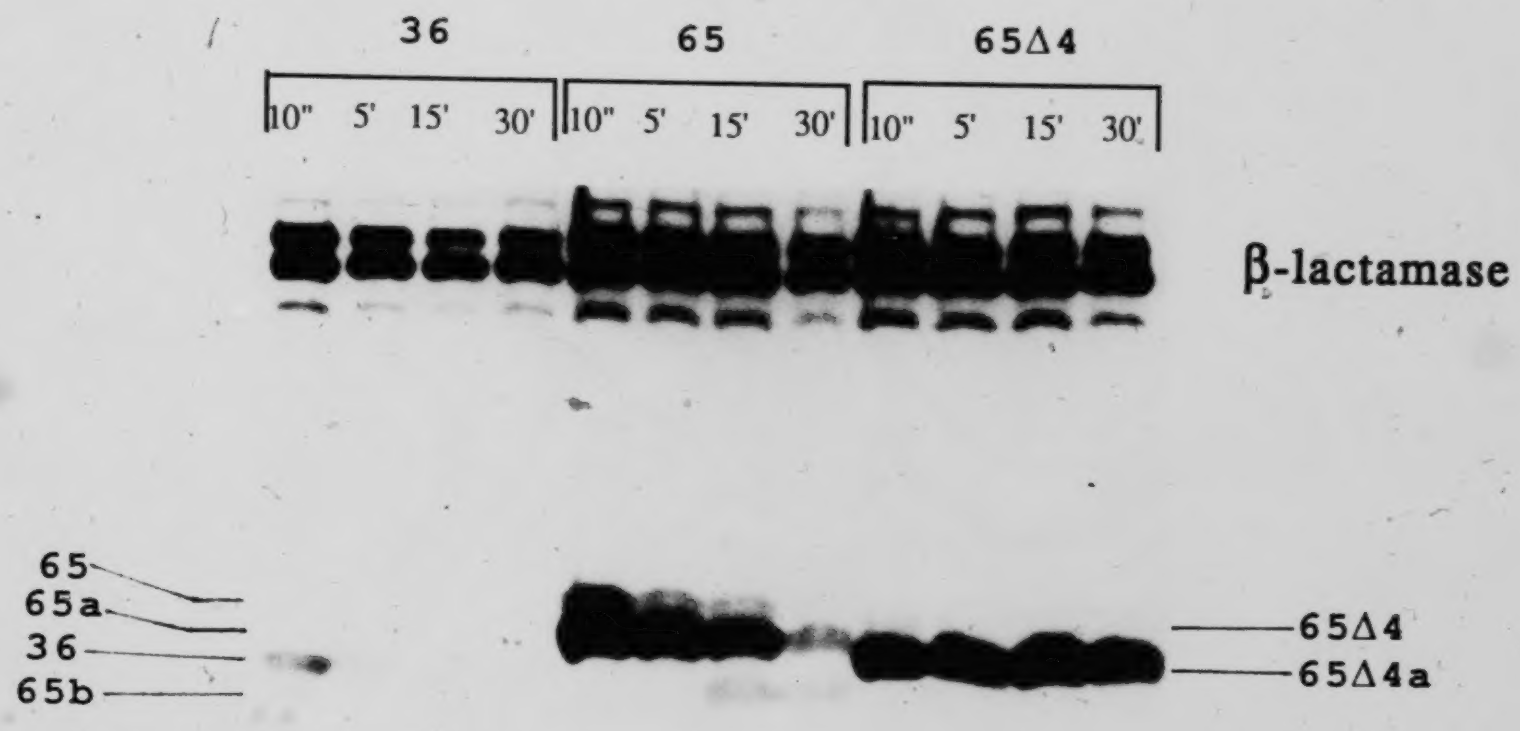
36
65
65Δ4
10"
5'
15'
30'
10"
5'
15'
30'
10"
5'
15'
30'
β-lactamase
65
65a
36
65b
65Δ4
65Δ4a

FIGURE V.9

Model to explain the pulse-chase data of peptides 65 and 65Δ4. Refer to the *Discussion* for the full explanation. The "pacmen" represent the membrane peptidases. The hatched oval indicates the cytoplasmic site of hydrolysis (site C) and the hatched rectangle symbolizes the periplasmic cleavage site (site P). Peptides 65_a and $65\Delta 4_a$ consist of the residues from the N-terminus to the hatched oval. Peptides 65_b and $65\Delta 4_b$ consist of the residues from the N-terminus to the hatched rectangle. The 4 residues deleted from peptide 65 are indicated by the +4 and +7. Note that the deletion mutation within peptide 65 makes site C more available to the cytoplasmic membrane peptidase. Also, site P of peptide 65Δ4 is made inaccessible to the periplasmic peptidase due to the deletion mutation.

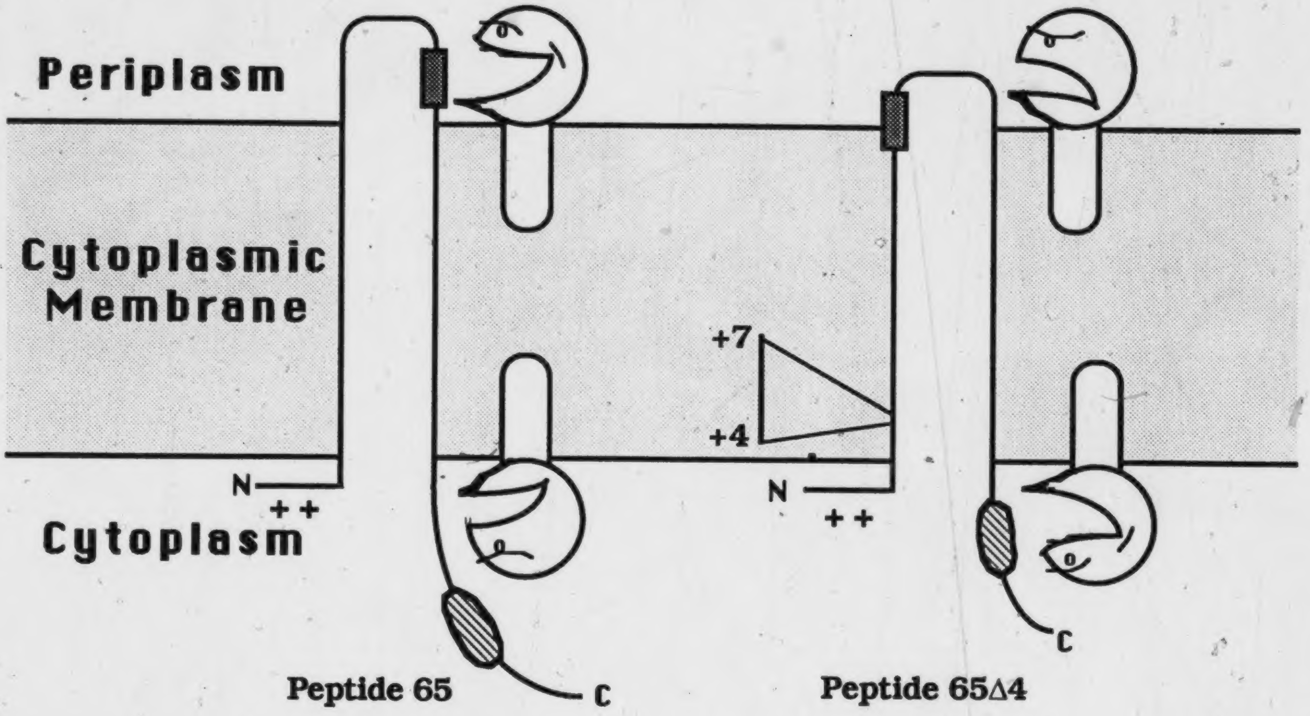
Periplasm
Cytoplasmic Membrane
Cytoplasm
N
++
C
Peptide 65
+7
+4
N
++
C
Peptide 65Δ4

TABLE V.1

The Chimeras Coding for the Mutant Pilins

pUC8 Chimeras	pT7-4 Chimeras	Translated product
pBP001	pBP009	prepilin
pBP007	pBP009	prepilin
pBP012	pBP014	prepilinΔ8
pBP013	pBP015	prepilinΔ4
pBP021	pBP031	peptide 65
pBP022	pBP032	peptide 65Δ8
pBP023	pBP033	peptide 65Δ4
pBP026	pBP036	peptide 36

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Chou, P.Y., and Fasman, G.D. (1978) Emperical predictions of protein conformation. *Ann Rev Biochem* **47**: 251-276.

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Randall, L. L. (1983) Translocation of domains of nascent periplasmic proteins across the cytoplasmic membrane is independent of elongation. *Cell* **33**: 231-240.

Rosenblatt, M., Beaudette, N.V., and Fasman, G.D. (1980) Conformational studies of the synthetic precursor-specific region of preproparathyroid hormone. *Proc Natl Acad Sci USA* **77**: 3983-3987.

Russel, M., and Model, P. (1984) Replacement of the *fip* gene of *Escherichia coli* by an inactive gene cloned on a plasmid. *J Bacteriol* **159**: 1034-1039.

Sastry, P.A., Finlay, B.B., Pasloske, B.L., Paranchych, W., Pearlstone, J.R., and Smillie, L.B. (1985a) Comparative studies of the amino acid and nucleotide sequences of pilin derived from *Pseudomonas aeruginosa* PAK and PAO. *J Bacteriol* **164**: 571-577.

Sastry, P. A., Pearlstone, J. R., Smillie, L. B., and Paranchych, W. (1985b) Studies on the primary structure and the antigenic determinants of pilin isolated from *Pseudomonas aeruginosa* K. *Can J Biochem Cell Biol* **63**: 284-291.

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Smith, M. (1985) *In vitro* mutagenesis. *Ann Rev Genet* **19**: 423-462.

Steiner, F.S., Quinn, P.S., Chan, S.J., Marsh, J., and Tager, H.S. (1980) Processing mechanisms in the biosynthesis of proteins. *Annals New York Academy of Sciences* **343**: 1-16.

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Watts, T.H., Kay, C.M., and Paranchych, W. (1983) Spectral properties of three quaternary arrangements of *Pseudomonas* pilin. *Biochemistry* **22**: 3640-3646.

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Chapter VI

THE EXPRESSION OF MUTANT PILINS IN *PSEUDOMONAS AERUGINOSA*: FIFTH POSITION GLUTAMATE AFFECTS PILIN METHYLATION[1]

A. INTRODUCTION

Pseudomonas aeruginosa encodes and secretes an arsenal of virulence factors such ~~as~~ elastase, phospholipase C, exotoxin A, and exoenzyme S (Woods, 1987). Included are the NMePhe pilins which are translated with a positively charged amino-terminal extension of 6 or 7 amino acids as determined by DNA sequencing and N-terminal sequencing (Pasloske *et al.*, 1988). Prior to or during pilus assembly, this leader peptide is removed and the resulting N-terminal phenylalanine (Phe) becomes methylated.

Proteins destined for export within prokaryotes are usually nascently expressed with an extra 15 to 30 residues at the N terminus (Michaelis and Beckwith, 1982). This extension, known as a signal sequence, contains one or more positively charged residues at the extreme N terminus, followed by a hydrophobic region and then a site of cleavage which is slightly polar (von Heijne, 1985). The first 36 residue region of prepilin is reminiscent of a typical signal sequence except that the cleavage site follows the positively charged sequence and not the hydrophobic region. In contrast, other exported proteins in *P. aeruginosa* such as

[1] A version of this chapter has been published. **Pasloske, B.L. and Paranchych, W.** 1988. Mol. Microbiol. 2: 489-495.

porin protein F, exotoxin A, and azurin (Duchene *et al.*, 1988; Lory *et al.*, 1988; Canters, 1987) have signal sequences of the conventional length, indicating that NMePhe pilin may follow a different pathway of transport in *P. aeruginosa.* To begin characterizing the relevant amino acids involved in pilin maturation, we created mutations within the conserved, N-terminal region of mature pilin and observed their affect upon leader peptide cleavage and methylation within *P. aeruginosa.* We found that deletions of 4 or 8 amino acids stongly decreased the efficiency of leader peptide hydrolysis and pilin methylation. As well, substitution of a lysine (Lys) for the glutamate at position 5 (Glu-5) did not affect pilin transport into the membrane or peptide cleavage but did inhibit methylation of the N-terminal Phe.

B. MATERIALS and METHODS

1. Bacterial Strains and Plasmids.

P. aeruginosa PAO1 (American Type Culture Collection 25247) was obtained from D.E. Bradley (Memorial University, St. John's, Newfoundland, Canada). *Escherichia coli* MM294 (Ruvken and Ausubel, 1981) and plasmids pKT210 (Bagdasarian *et al.*, 1981) and pRK2013 (Figurski and Helinski, 1979) were provided by Pamela Sokol (University of Calgary Health Sciences Centre, Calgary, Alberta, Canada). The plasmids pT7-4 (Tabor and Richardson, 1985), pBP009, pBP014, and pBP015 (Pasloske *et al.*, 1988) and *E. coli* JM83 (Viera and Messing, 1982) were described previously. *P. aeruginosa* strain PAKp is a lab strain, derived from *P. aeruginosa* PAK (American Type Culture Collection 25102), which does not express any detectable levels of pilin.

2. Media and Antibiotics.

Luria-Bertani (LB) medium was described previously (Maniatis *et al.*, 1982). *Pseudomonas* isolation agar (Difco, Detroit, Michigan, USA) was prepared as recommended. Antibiotic concentrations were 50 $\mu g\ ml^{-1}$ for both ampicillin and chloramphenicol and 300 $\mu g\ ml^{-1}$ for carbenicillin.

3. Membrane Fractionation.

One litre each of the *P. aeruginosa* PAO1 transconjugants were grown with shaking in the presence of chloramphenicol to an OD_{600}=0.8 at 37°C. The cells were harvested by centrifugation and the membranes fractionated as previously described (Hancock and Nikaido, 1978). The procedure was modified in that the cells were allowed to incubate for 20 min at room temperature after the addition of the deoxyribonuclease. This step was followed by freezing the cells with dry ice and thawing them at room temperature before the cells were passed through the French press. The IM and OM1 fractions (Hancock and Nikaido, 1978) were the membrane fractions recovered and used as the representative cytoplasmic and outer membrane fractions, respectively.

4. Immunoblotting.

Immunoblotting was performed as previously described (Pasloske *et al.*, 1988).

5. Protein Sequencing.

The PAK pilin and pilin mutants were sequenced using methods similar to Chapter V except that the proteins were transferred onto polyvinylidene difluoride membranes as according to Matsudaira (1987). Approximately 1 nmole of each of the cytoplasmic membrane samples were fractionated by 15% sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) before electroblotting for a 2 h period.

6. Recombinant DNA techniques.

DNA sequencing and recombinant DNA techniques were undertaken as outlined earlier (Pasloske *et al.*, 1985).

7. Nitrosoguanidine Mutagenesis of the PAK Pilin Gene.

E. coli JM83 transformed with pBP500 was grown to 40 Klett units (2×10^8 cells). They were harvested by centrifugation and resuspended in one half volume of 100 mM sodium citrate (pH 5.5). A 100 μl aliquot of 1 mg ml^{-1} nitrosoguanidine (dissolved in water) was added to 1 ml of the cells, followed by incubation at 37^{o}C for 30 min. The cells were diluted 100-fold in LB broth with chloramphenicol and ampicillin and were left standing overnight at 37^{o}C.

Mutant pilin genes were selected by transferring pBP500 from the overnight culture of *E. coli* JM83 into *P. aeruginosa* PAKp by triparental mating and purifying for pilus-specific, bacteriophage-resistant mutants. After the triparental mating, the cells on the filter were resuspended in LB broth, incubated with bacteriophage PO4 for 10 min at 37°C, and the *P. aeruginosa* transconjugants selected on carbenicillin-*Pseudomonas* isolation agar. Colonies which grew were assayed for PO4 sensitivity. The mutated pBP500 DNA was isolated from PO4-resistant cells and the pilin genes sequenced.

8. Plasmid Transfer by Conjugation.

Conjugal plasmid transfer from *E. coli* to *P. aeruginosa* was performed using the procedure similar to that of Goldberg and Ohman (1984). Donor and recipient cells were incubated together with *E. coli* MM294 which contains the helper plasmid pRK2013. The helper plasmid has all gene products necessary for plasmid conjugation with compatible plasmids. In a triparental mating, pRK2013 transfers itself into the donor cell and is then able to transfer the donor's plasmid which has an origin of transfer but lacks the other transfer genes needed for conjugation (Willetts, 1984). The triparental mating occurred on a Millipore membrane filter (0.45 µm pore size) incubated overnight at 30°C. The cells were resuspended in LB broth and the transconjugants selected on carbenicillin-*Pseudomonas* isolation agar.

9. Bacteriophage Sensitivity Assay.

P. aeruginosa transconjugants were streaked onto carbenicillin-LB agar and 2 µl of the pilus-specific bacteriophage PO4 (Bradley and Pitt, 1974) were pipetted onto the cells. After incubation overnight at 30°C, the transconjugants were checked for zones of clearing, indicating lysis by the PO4.

C. RESULTS

1. Cloning the Pilin Genes into the Broad-Host Range Vector, pKT210.

The plasmids pBP009, pBP014, and pBP015 are derived from the vector plasmid pT7-4 and contain DNA inserts encoding the PAK wild-type pilin gene and the genes expressing an 8 or a 4 amino acid deletion pilin product, respectively (see Table 1). pT7-4 is a vector encoding a T7 promoter which can be used in combination with another plasmid expressing a T7 polymerase to produce high levels of protein (Tabor and Richardson, 1985). For expression of these pilin genes in *P. aeruginosa*, it was necessary to clone them into a broad-host range vector such as pKT210 (Bagdasarian *et al.*, 1981). Each of the pT7-4 constructs and pKT210 were linearized with *Eco*RI, ligated together and the mixture transformed into *E. coli* JM83 (Fig. 1). Selection using chloramphenicol and ampicillin was used to isolate the desired recombinants. Digestion with *Hin*dIII determined that the direction of pilin transcription was in the same direction as the chloramphenicol resistance gene, thus producing the chimeras listed in Table 1. The complete protein sequence of PAK pilin is found in Pasloske *et al.* (1985) while the pertinent 4 and 8 amino acid deletions within the PAK pilin were presented recently (Pasloske *et al.*, 1988).

2. NTG Mutagenesis Selecting for Pilin Mutants.

N-methyl-*N*-nitroso-*N'*-nitrosoguanidine (NTG) mutagenesis of pBP500 was performed as outlined in *Materials and Methods*. One of the mutants resistant to bacteriophage PO4 was sequenced and it was discovered that the codon of GAA coding for glutamate at position 5 (Pasloske *et al*., 1988) was mutated to an AAA which coded for lysine. This clone was named $pBP500_9$ and the expressed pilin product was called pilin Glu-5-to-Lys (Table 1).

3. Membrane Fractionation.

The chimeras listed in Table 1 were mobilized into *P. aeruginosa* PAO1 and the cytoplasmic and outer membranes of each transconjugant were prepared as described in *Materials and Methods*. These membranes were subjected to 15% SDS-PAGE and stained with Coomassie blue (Fig. 2A). The immunoblot of an equivalent gel, with anti-PAK pili serum (Fig. 2B) detected PAK pilin within the transconjugants harboring the cloned PAK pilin genes (pBP500, pBP540, pBP580, and $pBP500_9$). In contrast, the anti-PAK pili serum did not cross-react with the chromosomally encoded PAO1 pilin expressed by cells containing the pBP400 control vector even though anti-PAO pili serum detected PAO pilin in each of the transconjugants (data not shown). In Fig. 2A, note that the plasmid-encoded PAK pilins were in greater amounts than the chromosomally encoded PAO1 pilin (except for pilinΔ8) in the cytoplasmic membrane fractions. The fact that the PAK pilins were not observed in the outer membrane fractions in Fig. 2A indicates that the pilin is pooling primarily in the cytoplasmic membrane.

It was not possible to predict the state of the N termini of the different pilins by comparing their mobilities in SDS-PAGE because of the anomalous migrations of the pilins due to the mutations. Therefore, to assess the degree of processing of the PAK pilins within the membranes, the N termini of the pilins were sequenced from the cytoplasmic membrane fractions as outlined in *Materials and Methods*. The results of the sequencing are summarized in Table 2.

The wild-type PAK pilin was cleaved correctly at the Gly-Phe peptide bond and the Phe methylated while the deletion of 8 amino acids completely abolished processing, thus preventing methylation. Only about 10% of the pilinΔ4 was proteolytically cleaved, as determined by amino acid sequencing. However, those pilins which were cleaved were not methylated. Interestingly, the Glu-5-to-Lys mutation did not prevent leader peptide removal but it completely inhibited the methylation of the N-terminal Phe.

D. DISCUSSION

In prokaryotes, proteins to be transported outside the bounds of the cytoplasmic membrane are translated with an amino-terminal extension of 15 to 30 amino acids. Usually this signal sequence peptide can be structurally separated into 3 major regions: (i) a positively charged N-terminus; (ii) a central core of hydrophobic amino acids; and (iii) a more polar C-terminal region in the vicinity of the cleavage site (von Heijne, 1985). The first 36 residues of the prepilin from *P. aeruginosa* PAK are characteristic of a conventional signal sequence except that the proteolytic site falls between regions (i) and (ii). The enzyme responsible for this cleavage is probably in a class separate from the other characterized signal peptidases since the sequence surrounding the pilin cleavage site is distinctly different from that observed for the signal peptide cleavage sequence.

As well, pilin is not fully mature until it has been methylated at the N-terminal Phe. This particular modification appears to be a relatively unique phenomena of the NMePhe pilins, although Stock *et al.* (1987) have listed 10 other prokaryotic and eukaryotic proteins with different N-terminal amino acids which are also methylated at their N termini. It is likely that the methyltransferase of the NMePhe pilins is quite distinct from the other methyltransferases since their N-terminal sequence is distinct from any of the other N-termini of the N-methylated proteins (Stock *et al.*, 1987). In short, maturation of the NMePhe pilins appears to be a two part process requiring two unique enzymes. This suggests that the mode of pilin secretion may represent a unique pathway.

To begin characterizing the specific sequences required for each distinct processing event, *in vivo* analysis of the expression of mutant pilin genes in *P. aeruginosa* was undertaken. A deletion of Ile-4 to Met-7 reduced the efficiency of leader peptide cleavage by 90% while the removal of Ile-4 to Ala-11 increased the inhibition of peptide cleavage and markedly decreased partioning into the cytoplasmic membrane. Several reasons can be postulated for the effects observed from these deletions. First, it is possible that specific residues were deleted which the leader peptidase recognizes and therefore substrate recognition was poor. Second, the deletion may have incorrectly positioned the peptidase site by shortening the hydrophobic stretch of amino acids. This hypothesis is supported by the results of Pasloske *et al.* (1988) which showed that the deletion of the same 4 amino acids could affect the efficiency of peptidases on sites as many as 23 residues from the deletion. Third, the secondary structure at the site of the deletion may have been altered, thus affecting leader peptidase recognition of the cleavage site. Calculations (not shown) of secondary structure predictions indicate that the deletions increased the beta-sheet potential and diminished the alpha-helix potential of this region.

Previous studies on the expression of the same deletion mutants in *E. coli* produced similar results (Pasloske *et al.*, 1988). While overexpression of the pilins resulted only in the detection of the unprocessed forms, expression of the pilin genes under a weaker promoter revealed a slight modification in the processing of pilin. The wild-type pilin was detected in two forms, the molecular weights corresponding to the processed and unprocessed forms while the pilinΔ4 and pilinΔ8 only appeared in their unprocessed forms. Therefore, these deletions inhibited the proteolysis of pilin in *E. coli* just as they did in *P. aeruginosa* suggesting that *E. coli* may have a protease weakly related to the leader peptidase of *P. aeruginosa*.

Besides abolishing leader peptide cleavage, the 8 amino acid deletion destabilized the pilin since it was not detected by Coomassie blue staining in the cytoplasmic membrane fractions whereas the other pilins were visible (Fig. 2A). This data correlates with the results in *E. coli* of pilinΔ8 expression. PilinΔ8 was not found in the same amounts as pilin and pilinΔ4 in the cytoplasmic membrane when the pilins were overproduced using a T7 polymerase/promoter system (Pasloske *et al.*, 1988). It is likely that the deletion made pilin transport incompetent and much of the pilin was degraded in the cytoplasm. As Ferenci and Silhavy (1987) proposed, the N-terminal region of the mature protein may be necessary to interact with various proteins for efficient transport or possibly the deletion weakened the required secondary structure of the N terminus for transport into the membrane. The other possibility is that pilinΔ8 was partitioned efficiently into the membrane but then was quickly degraded.

Our data indicated that peptide hydrolysis and methylation are two independant events. The results of experiments with the pilin Glu-5-to-Lys mutant demonstrated that leader peptide removal was possible without concomitant methylation. In other words, methylation was not an obligatory result of peptide cleavage. We can not refute the remote possibility that the pilin was methylated only to be rapidly demethylated by an antagonizing enzyme which recognizes the N-terminal sequence of Glu-5-to-Lys pilin. We can only argue that the existence of such an enzyme would be highly coincidental and thus, we maintain that Glu-5 is involved in the recognition site of the putative transmethylase.

As shown in Fig. 2A, the majority of the pilin was found in the cytoplasmic membrane although a small amount of pilin was detected in the outer membrane as well, as previously reported by Watts *et al.* (1982). It is interesting that the unprocessed forms of pilinΔ4 and pilinΔ8 were detected in the outer membrane as readily as the processed wild-type pilin. Processing does not appear to be required for pilin to reach the outer membrane. An equilibrium may exist between the two membranes with pilin flowing freely through adhesion zones (Bayer, 1975). The preference of pilin for the cytoplasmic membrane suggests that the site of assembly may originate from the cytoplasmic membrane. This idea is analogous to the F pilus system of *E. coli* in which F pili have been visualized as emerging from adhesion zones (Bayer, 1975).

In summary, these data demonstrate that the mature sequence of pilin encodes information for the efficient processing and export of pilin. The idea that a mature protein sequence possesses "positive information" for transport was recently documented in the case of LamB in which the first 28 amino acids of the mature protein were demonstrated to be involved in the efficient export of LamB (Ferenci and Silhavy, 1987). NMePhe pilin is unique in that, not only does the mature N-terminus encode "positive information" for export but it also has an important functional role in the assembly of pili (Watts *et al.*, 1983). Experiments are now in progress to determine what affects these pilin mutants have on the polymerization of pili.

FIGURE VI.1

Construction of the plasmids for the expression of the pilin genes. Both the vector pKT210 and the pT7-4 constructs were linearized with *Eco*RI and then ligated together to acquire both chloramphenicol (Cm) and carbenicillin (Cb) resistance. The arrows represent the position and the direction of transcription of the genes indicated. The bold arrow indicates the pilin gene. Refer to Table 1 for the pilin product associated with each chimera.

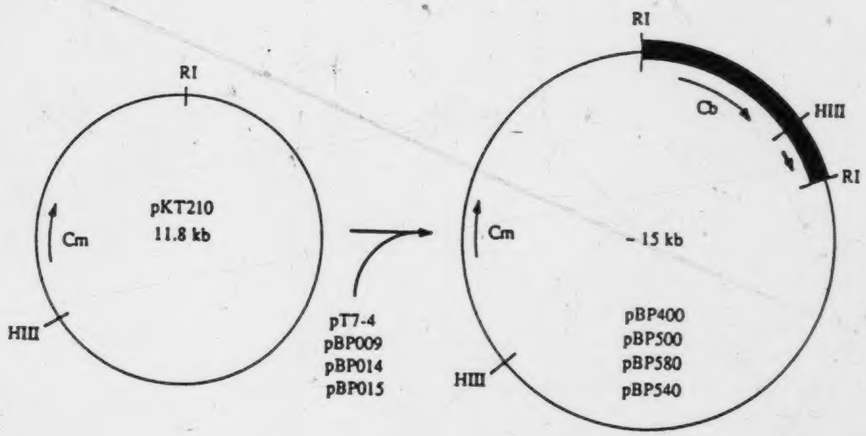
RI
pKT210
11.8 kb
Cm
HIII
pT7-4
pBP009
pBP014
pBP015
RI
Cb
HIII
RI
Cm
~ 15 kb
pBP400
pBP500
pBP580
pBP540
HIII

FIGURE VI.2

Membrane fractionations and immunoblot of the *P. aeruginosa* expressing the mutant pilin genes. Approximately equal amounts of the cytoplasmic and outer membrane fractions of the transconjugants of *P. aeruginosa* PAO1 expressing the various PAK pilin genes were subjected to 15% SDS-PAGE. A. Coomassie blue stained gel of the membrane fractions and 4 µg of PAK pilin as a standard. B. An immunoblot of the gel in (A) using anti-PAK pili serum except that only 0.2 µg of PAK pilin was loaded as a control. The molecular weight ($x10^{-3}$) standards are on the left-hand side of the figures.

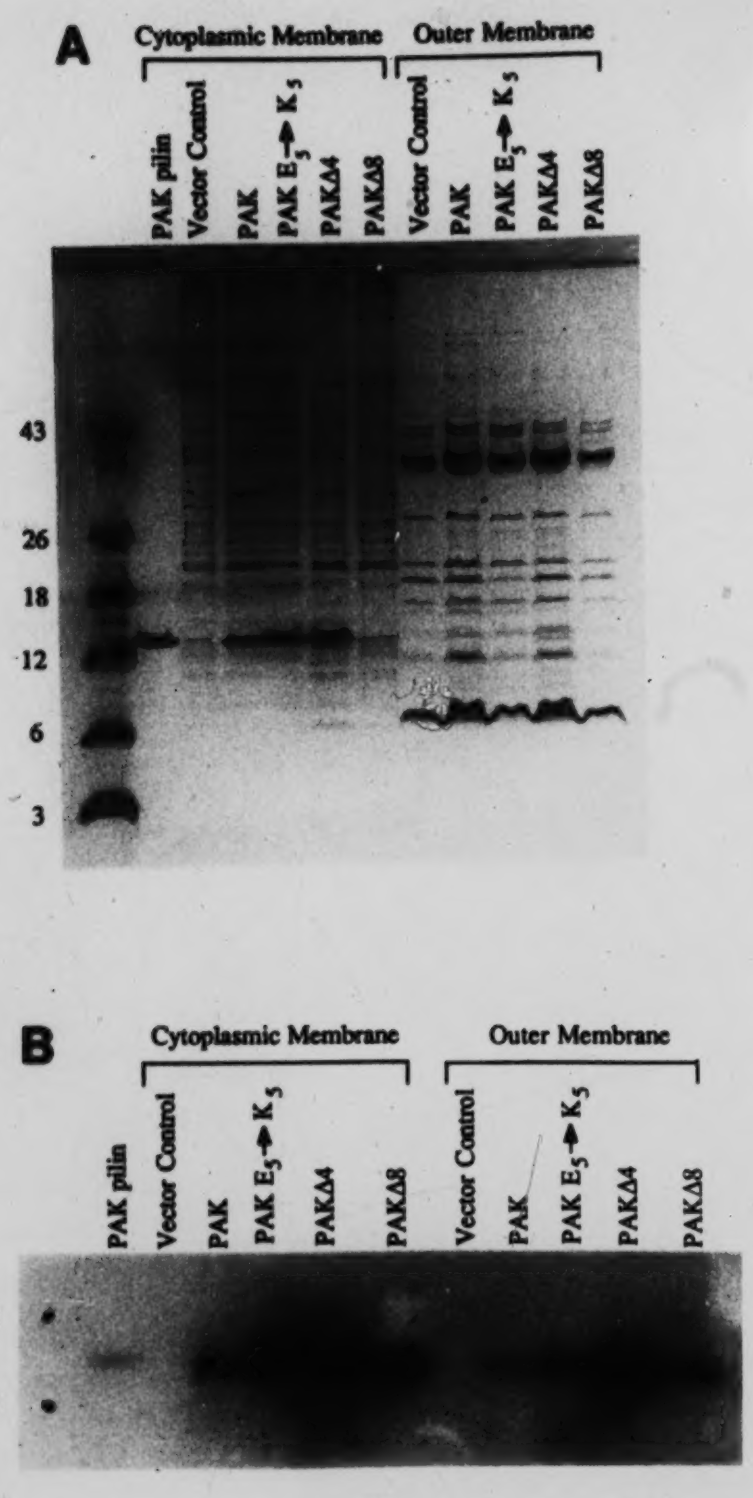
A
Cytoplasmic Membrane
Outer Membrane
PAK pilin
Vector Control
PAK
PAK $E_5 \rightarrow K_5$
PAKΔ4
PAKΔ8
Vector Control
PAK
PAK $E_5 \rightarrow K_5$
PAKΔ4
PAKΔ8
43
26
18
12
6
3
B
Cytoplasmic Membrane
Outer Membrane
PAK pilin
Vector Control
PAK
PAK $E_5 \rightarrow K_5$
PAKΔ4
PAKΔ8
Vector Control
PAK
PAK $E_5 \rightarrow K_5$
PAKΔ4
PAKΔ8
18
12

TABLE VI.1

The Chimeras Encoding the Mutant Pilins.

pT7-4 constructs[a]	pKT210 chimeras	PAK pilin expressed
pT7-4	pBP400	--- (control vector)
pBP009	pBP500	pilin wt
pBP015	pBP540	pilinΔ4[b]
pBP014	pBP580	pilinΔ8[c]
---	$pBP500_9$	pilin Glu-5-to-Lys

a. Pasloske *et al.*, 1988.

b. Deletion of amino acids Ile_4 to Met_7.

c. Deletion of amino acids Ile_4 to Ala_{11}.

TABLE VI.2

Characterization of the N-Termini of the Mutant Pilins Isolated from the Cytoplasmic Membrane Fractions.

Pilin product	Prepilin	Pilin(unmethylated)	Pilin(methylated)
Pilin wt	-	-	+
Pilin∆4	+	+	-
Pilin∆8	+	-	-
Pilin Glu-5-to-Lys	-	+	-

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Chapter VII

ASSEMBLY OF MUTANT PILINS IN *PSEUDOMONAS AERUGINOSA*: FORMATION OF PILI COMPOSED OF HETEROLOGOUS SUBUNITS[1]

A. INTRODUCTION

The highly conserved N termini of the NMePhe pili suggests that this region is involved in an important function(s). It is likely that the N terminus acts as a signal sequence for membrane targeting. Another possibility is that the necessary stabilizing forces for subunit-subunit interaction during pilus assembly are provided by this part of the molecule (4, 15). Recently, mutations were made within the N-terminal region of the pilin gene and their effects on pilin stability and processing within *P. aeruginosa* were determined (12). Deletions of 4 or 8 residues (amino acids 4 to 7 or 4 to 11, respectively) abolished leader peptide cleavage; however, substitution of a lysine (Lys) for glutamate at position 5 (Glu-5) in mature pilin resulted in complete leader peptide removal without any detectable methylation of the N-terminal phenylalanine.

This manuscript describes the effects that these pilin mutations have upon pilus assembly. For example, pilins with deletions of 4 or 8 amino acids were not incorporated into pili at all. In contrast, pilin having the Glu-5-to-Lys mutation was incorporated heterogeneously into pili in the presence of wild-type pilin but it was unable to assemble as a homopolymer. When viewed by the electron microscope, these mixed pili exhibited a corkscrew-like morphology as compared to the straight filaments of wild-type pili. These observations indicate that the NMePhe or Glu-5 or both amino acids play an intergral role in the polymerization of the pilin subunits.

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[1] A version of this chapter has been published. Pasloske, B.L., Scraba, D.G. and Paranchych, W. 1989. J. Bacteriol. 171: 2142-2147.

B. MATERIALS and METHODS

1. Bacterial Strains, Plasmids, and Growth Media.

The bacterial strains and plasmids used are listed in Table 1. The media used were Luria-Bertani (LB) medium (9), *Pseudomonas* isolation agar, and MacConkey Agar (MA). *Pseudomonas* isolation agar and MA were purchased from Difco, Detroit, Michigan, USA. Lactose was added to 0.3% (wt/vol) in MA agar. Antibiotic concentrations were as follows: mercuric chloride (Hg), 15 μg ml^{-1}; chloramphenicol and streptomycin, 200 μg ml^{-1}; nalidixic acid, 40 μg ml^{-1}; carbenicillin, 300 μg ml^{-1}; tetracycline, 10 and 150 μg ml^{-1} for *E. coli* and *P. aeruginosa*, respectively.

2. Pili Preparations.

The transconjugants of *P. aeruginosa* DB2 were grown on 8 cookie sheets (27 by 390 cm) in LB agar overnight at 30°C. The cells were scraped off into 150 ml of 1x SSC buffer (150 mM NaCl plus 15 mM sodium citrate, pH 7.0) and subjected to vigorous mixing with a magnetic stirrer at 4°C for 1 h. The cells were centrifuged several times at 6000 x *g* for 10 min until a pellet was no longer visible after centrifugation. The pH of the supernatant was adjusted to 4.3 with 1 M citric acid. The pili were allowed to precipitate at 4°C for 4 h, subjected to centrifugation (6000 x *g*) for 15 min, and resuspended in 0.05 M phosphate buffer (pH 7.0).

3. Phage Sensitivity Assay.

The *P. aeruginosa* transconjugants were streaked onto tetracycline-LB agar and 2 µl of the pilus-specific bacteriophage PO4 (1×10^9 PFU/ml) (3) were pipetted onto the center of the streak. The cells were incubated overnight at 30°C. A clear zone within the growing cells indicated lysis and the presence of retractile pili.

4. Immunoblotting.

Immunoblotting was performed as previously described (10).

5. Plasmid Transfer by Conjugation.

Triparental matings were performed as described in Chapter VI.

In order to transfer the F *lac* plasmid by conjugation, donor and recipient strains were each grown overnight without shaking at 37°C in 2 ml of LB broth. The mating involved adding 0.2 ml of the donor and 0.2 ml of the recipient strains to 2 ml of LB broth and incubating them at 37°C for 30 min. The mating solution was plated onto the appropriate selective medium and incubated at 37°C.

6. Electron Microscopy and Immunolabeling.

The transconjugants of *P. aeruginosa* DB2 were grown on chloramphenicol-LB agar overnight at 30°C. A loopful of the cells was resuspended in 50 µl of phosphate-buffered saline, pH 7.0, and 5 µl of the suspension was placed onto a hydrophilic carbon film supported by a copper mesh grid. If the cells were not incubated with antibody, they were then washed twice with reticulocyte standard buffer (10 mM Tris hydrochloride, 10 mM NaCl, 1.5 mM $MgCl_2$, pH 7.0) and stained with 0.75% sodium phosphotungstate (pH 7.0).

If the cells were to be coated with antibody, a 1:50 dilution of either anti-PAK or anti-PAO pili serum in phosphate-buffered saline was added to the cells on the grid and incubated for 5 min at room temperature. The grid was then washed twice with reticulocyte standard buffer and stained with 0.75% sodium phosphotungstate.

The grids were examined with a Philips EM240 electron microscope.

7. Protein Sequencing.

N-terminal sequencing was performed as in Chapter VI with the following modifications. Purified pili were solubilized by adding approximately a 0.5x volume of 0.1 M ammonium hydroxide. A 1-cm square of polyvinylidene difluoride membrane (Millipore Corp., Bedford, Mass.) was soaked with 100% methanol and rinsed with distilled water for 5 min. The membrane was placed on blotting paper, 2 nmole of pili was applied to the membrane and allowed to completely dry. The membrane was rewetted with 50% methanol for a few seconds and then rinsed in distilled water over a 24 h period with the water being changed every few hours. The membrane was dried, and automated Edman degradation performed as previously described (10).

8. Transposition of Tn*501* into the Chromosome of *E. coli* HB101.

Tn*501*::RSF1010 was transformed into *E. coli* ED2602, and red colonies were selected on Hg-lactose-MA agar. The *E. coli* ED2602 (Tn*501*::RSF1010) was mated with *E. coli* CSH56 and red transconjugants were selected on naladixic acid-Hg-lactose-MA plates. The *E. coli* CSH56 (Tn*501*::F *lac*) was crossed with HB101 and red transconjugants were selected on Hg-streptomycin-lactose-MA medium. Spontaneous loss of the F *lac* plasmid with a coincidental integration of Tn*501* into the chromosome of *E. coli* HB101 was acheived by growing the *E. coli* HB101 (Tn*501*::F *lac*) in LB broth without antibiotic selection, then plating these cells onto Hg-streptomycin-lactose-MA agar and looking for white colonies. One white colony was selected which had the required phenotype and was named *E. coli* BLP1.

9. Cloning a Cosmid Containing the *P. aeruginosa* PAK Pilin Gene.

A cosmid bank of *P. aeruginosa* was prepared as outlined by Goldberg and Ohman (7), except that a Packagene Lambda DNA Packaging System (Bio/Can Scientific, Mississauga, Ontario, Canada) was used to package the cosmid DNA. The cosmid bank of *P. aeruginosa* PAK harbored in *E. coli* HB101 was plated on tetracycline-LB agar at a dilution sufficient to yield single, discrete colonies. Nitrocellulose filters were laid over the colonies then lifted off the plates. The filters with the cosmid bank were probed with the nick-translated 1.2-kilobase *Hind*III fragment containing the PAK pilin gene. One colony was detected harboring a cosmid (pBP310) having a 7-kilobase *Eco*RI fragment containing the pilin gene. This *Eco*RI fragment was the same size as that detected in Southern blots of the PAK genome digested with *Eco*RI (11). In total, pBP310 had seven *Eco*RI fragments, adding up to approximately 26 kilobases, cloned into the *Eco*RI site of pLAFR.

10. Construction of BLP4, a Pilin-Lacking Derivative of *P. aeruginosa* DB2.

pBP310 was transformed into *E. coli* BLP1 and then mobilized into *P. aeruginosa* PAKp. Transconjugants which grew on tetracycline-Hg-*Pseudomonas* isolation agar plates were tested for their sensitivity to the pilus-specific bacteriophage PO4. *P. aeruginosa* PAKp is resistant to PO4 but was complemented for phage sensitivity by pBP310. Transconjugants resistant to the phage were candidates for cosmids with a disrupted pilin gene. Cosmid DNA (pBP311) was purified from one isolate which was phage resistant and transformed into *E. coli* HB101. Restriction mapping indicated that Tn*501* had inserted into the 1.2-kilobase *Hin*dIII fragment of pBP310 containing the pilin gene. Further analysis by DNA sequencing (11) revealed that the transposon had integrated between the Phe-1 and Thr-2 codons of the pilin gene.

A gene replacement of the chromosomal PAO pilin gene with the disrupted PAK pilin gene was completed by performing a triparental mating of *E. coli* HB101 (pBP311) and *P. aeruginosa* DB2 and selecting transconjugants on Hg-*Pseudomonas* isolation agar medium. The colonies of multipiliated *P. aeruginosa* DB2 are small compared to the wild-type strain *P. aeruginosa*. Approximately 10% of the transconjugants were much larger than the other colonies, and these large colonies had lost their tetracycline resistance while the small colonies had not. The loss of tetracycline resistance indicated that pBP311 had been lost from the host. Direct examination of one of these large colonies (*P. aeruginosa* BLP4) by electron microscopy confirmed that the cells did not have any pili. The strongest evidence for homologous recombination was provided by restriction mapping of the total DNA from these isolates. Four different restriction digests of genomic DNA from the mutant isolates were probed with the PAK pilin gene probe. The mutant restriction patterns differed from the equivalent wild-type patterns and produced the fragment sizes expected of chromosomal DNA having undergone the anticipated exchange of the

Tn501-mutated PAK pilin gene for the PAO pilin gene (data not shown).

C. RESULTS

1. Electron Microscopy of *P. aeruginosa* PAO1-leu Expressing the Mutant Pilins.

The chimeras containing the different PAK pilin genes were mobilized into *P. aeruginosa* PAO1-leu and each transconjugant was examined for piliation by electron microscopy. The strains containing the vector control (pBP400) and the PAK pilin clone (pBP500) assembled pili; however, the pBP500 strain produced two- to threefold more pili (about 3 pili per cell) than the PAO background strain containing the vector alone. Transconjugants carrying pBP540 or pBP580 were unpiliated. These plasmids carry deletions that eliminate residues 4 to 7 (Δ4; pBP540) or 4 to 11 (Δ8; pBP580) of the mature pilin polypeptide. The pBP580 clone was quite lethal to *P. aeruginosa* PAO1-leu, resulting in about a 10,000-fold reduction in the number of transconjugants. The majority of the pBP500_9 transconjugants were bald although some cells (~5%) produced one or two very short pili (data not shown).

2. Electron Microscopy of the Multipiliated and Pilin-Lacking *P. aeruginosa* Strains DB2 and BLP4 Expressing the Mutant Pilins.

The same PAK pilin mutant constructs were mobilized into the multipiliated strain *P. aeruginosa* DB2. As observed by electron microscopy, the piliation of these strains differed from those seen with strain PAO1-leu. Transconjugants containing pBP400, pBP500, pBP540, and $pBP500_9$ were all multipiliated whereas it was not possible to procure a viable *P. aeruginosa* harboring pBP580. It is likely that the pilinΔ8 gene product was lethal to the multipiliated mutant. To establish the pilus composition of each of the transconjugants, the cells were incubated in the presence of rabbit anti-PAK or -PAO pili serum and observed by electron microscopy. The results are summarized in Table 2.

As expected, the pBP400 transconjugant had pili which adsorbed only anti-PAO pili serum. However, regardless of whether anti-PAK or -PAO pili serum was used, immunolabeling of the pBP500 transconjugant resulted in two types of pili: those coated with antibody along their entire length and those not coated at all (Fig. 1). The predominant serotype observed was PAK. Antibody-coated pili were easily distinguished from naked pili in the micrographs. These data indicate that each pilus was a homopolymer, composed of either PAK or PAO subunits arising from the cloned PAK or chromosomally encoded PAO pilin genes, respectively.

Pili expressed by transconjugants carrying pBP540 were coated with anti-PAO pili serum but not anti-PAK pili serum, suggesting that these pili consisted only of the chromosomally encoded PAO pilin subunits (data not shown). N-terminal sequence results for pili purified from this strain were consistent with this observation. A single NMePhe peak was detected in the first cycle of sequencing and only Glu was observed in the fifth cycle. Thus, the only detectable subunit was PAO pilin.

The pili of the pBP500$_9$ (Glu-5-to-Lys pilin) transconjugants had a wavy, braided appearance (Fig. 2A), not typical of wild-type PAO pili. These pili interacted with both anti-PAK and -PAO pili sera, although the anti-PAK pili serum coated the pili more heavily than the anti-PAO pili serum (Fig. 2B and C). These results suggest that each individual pilus contained both PAK and PAO subunits. N-terminal amino acid analysis of the pili indicated that unmethylated phenylalanine and NMePhe were present in a 3 to 1 ratio, respectively. Therefore, it is highly probable that phenylalanine originated from the unmethylated mutant PAK pilin incorporated into the same pili as the PAO subunit in a 3 to 1 ratio, respectively. Both glutamate and lysine were detected in the fifth cycle of the sequence analysis.

When the same clones were mobilized into the pilin-lacking *P. aeruginosa* BLP4, only bacteria containing pBP500 expressed pili; those containing pBP400, pBP540, and pBP500$_9$ were bald. It was not possible to grow a pBP580 derivative of this *P. aeruginosa* strain.

3. Immunoblots of the Purified Pili.

Purified pili were prepared from cultures of *P. aeruginosa* DB2 transconjugants containing pBP500, pBP540, and pBP500$_9$ grown without antibiotic present in medium. Immunoblots from the pBP500 and pBP540 strains reacted equally well with anti-PAO pili serum whereas the anti-PAK pili serum reacted weakly (Fig. 3A).

The weak reactivity of the pili from the pBP500 cells with the anti-PAK pili serum did not agree with the immunolabeling experiments (Fig. 1). Therefore, pili were purified from pBP500 cells grown in the same medium which had been used for the immunolabeling experiments, that is, in the presence of chloramphenicol. Immunoblots of these pili showed strong reactivity with the anti-PAK pili serum and weak reactivity with the anti-PAO pili serum, thus duplicating the immunolabeling results (Fig. 3B).

The purified pili from the $pBP500_9$ transconjugant reacted more strongly with anti-PAK pili serum than with the anti-PAO pili serum which was consistent with the 3 to 1 ratio of PAK to PAO pilin determined by the N-terminal sequencing of the purified pili.

D. DISCUSSION

Recent experiments revealed that deletions within the highly conserved N terminus of *P. aeruginosa* NMePhe pilin resulted in poor leader peptide cleavage; a Glu-to-Lys mutation at position 5 resulted in a loss in detectable methylation of the N-terminal phenylalanine, although leader peptide removal was not affected (12). To assess the effect of these pilin mutations on pilus assembly, the piliation of various transconjugants was observed by electron microscopy and the compositions of the pili were determined by three different methods (immunoblotting and N-terminal sequencing of purified pili, and the immunolabeling of pili on whole cells).

The mutant pilin clones were mobilized into three different strains of *P. aeruginosa* and each strain of transconjugant was examined. The pBP400 (control vector) transconjugants behaved as expected, producing only endogenous pili (or no pili at all in the pilin-lacking mutant). In all three hosts harboring the PAK pilin clone (pPB500), not only was the wild-type PAK subunit properly processed in the membrane but it was assembled into pili in a host which normally assembles PAO pili. This was not surprising since it has recently been demonstrated that *B. nodosus* pili are assembled in *P. aeruginosa* DB2 when *B. nodosus* pilin subunits are provided (5). These data demonstrate that the ancillary factors involved in NMePhe pilus assembly have a broad specificity for NMePhe pilins. Since the homology of *P. aeruginosa* pilin with the *B. nodosus* pilin is essentially confined to the first 30 residues, and PAK and PAO subunits have about as much overall homology with each other as they do with the *B. nodosus* pilins, it is likely that the conserved N-terminal sequence plays an important role in NMePhe subunit assembly.

Immunolabeling revealed that each pilus of *P. aeruginosa* DB2 (pBP500) was homogeneous, that is, each pilus was composed entirely of either PAK or PAO pilin. This phenomenon was previously observed in the case of two serotypically different *B. nodosus* pilins assembled in *P. aeruginosa* DB2 (5). Elleman and Peterson hypothesized that the pili were homogeneous because translation and assembly were closely linked temporally and spatially. As a result, one mRNA molecule might produce all the pilin needed for one pilus at a single assembly site. However, our observation that the Glu-5-to-Lys mutant PAK pilin was assembled into heterologous PAO pili contradicts this concept. Our data indicate that a pilin pool localized in the membranes of *P. aeruginosa* (12, 16) can be utilized for pilus assembly. They further suggest that the key component to the interaction of NMePhe subunits is the conserved N-terminal sequence since two subunits with only 65% overall homology to each other were able to assemble into a pilus. It is interesting that the Glu-5-to-Lys mutant pilin assembled in the presence of the wild-type PAO pilin into mosaic pili but was incapable of self-polymeriztion in the pilin-lacking mutant. It is not clear from these results whether it is the Glu-5 or NMePhe or both residues which are critical to pilus formation.

The inability of the pilinΔ4 subunits to assemble in the pilin-lacking host (*P. aeruginosa* BLP4) was consistent with the observation that only PAO pilin was assembled in *P. aeruginosa* DB2 (pBP540). Even though a small proportion of the pilinΔ4 subunits have their leader peptides cleaved (12), the deletion in the N terminus still leads to aberrant assembly. Therefore, it is likely that the deleted residues are required either for recognition by the assembly machinery or for stabilization of subunit interactions.

Assembly of each of the mutant PAK pilins was tested in the presence of the wild-type PAO pilin but not in the presence of the wild-type PAK pilin. Lacking the antisera which could distinguish between wild-type and mutant PAK pilin subunits within the assembled pilus by the immunolabeling strategy, it was not feasible to do the same experiments with the wild-type PAK pilin. However, we suspect that results similar to the PAO studies would be obtained using the PAK wild-type pilin subunit.

In *P. aeruginosa* PAO1-leu, essentially no pili were assembled from the Glu-5-to-Lys mutant, whereas in the multipiliated strain DB2 this mutant pilin was efficiently assembled. This observation might be explained by a concentration-dependant model for pilus assembly. Grasberger *et al.* (8) postulated a theoretical membrane-protein system in which the probability of interactive species associating is enhanced by increasing protein concentrations in the membrane. This model does not exclude the possibility that pilin translation and assembly are closely linked (5), in which case, pilin levels would be high due to the localization of nascently translated pilin from single mRNA transcripts at the assembly sites. If the pilin subunit was inherently incapable of self-polymerization as in the case of the Glu-5-to-Lys mutant pilin, assembly would not be linked to translation. This would lead to an increased pilin concentration in the membrane. In the multipiliated strain, the mutant pilin concentration would reach the critical level for polymerization postulated by Grasberger *et al.* and pili would assemble provided PAO pilin was present to catalyze the reaction. However, in *P. aeruginosa* PAO1-leu, the pilin would not reach the necessary concentration for assembly to occur.

FIGURE VII.1

Immunolabeling of the pili assembled by the transconjugants of *P. aeruginosa* DB2 carrying pBP500. *P. aeruginosa* DB2 harboring pBP500 were incubated with either anti-PAO (A) or -PAK (B) pili serum, negatively stained, and observed with the electron microscope. The single arrows point to a naked pilus, double arrows point to a pilus coated with antibody.

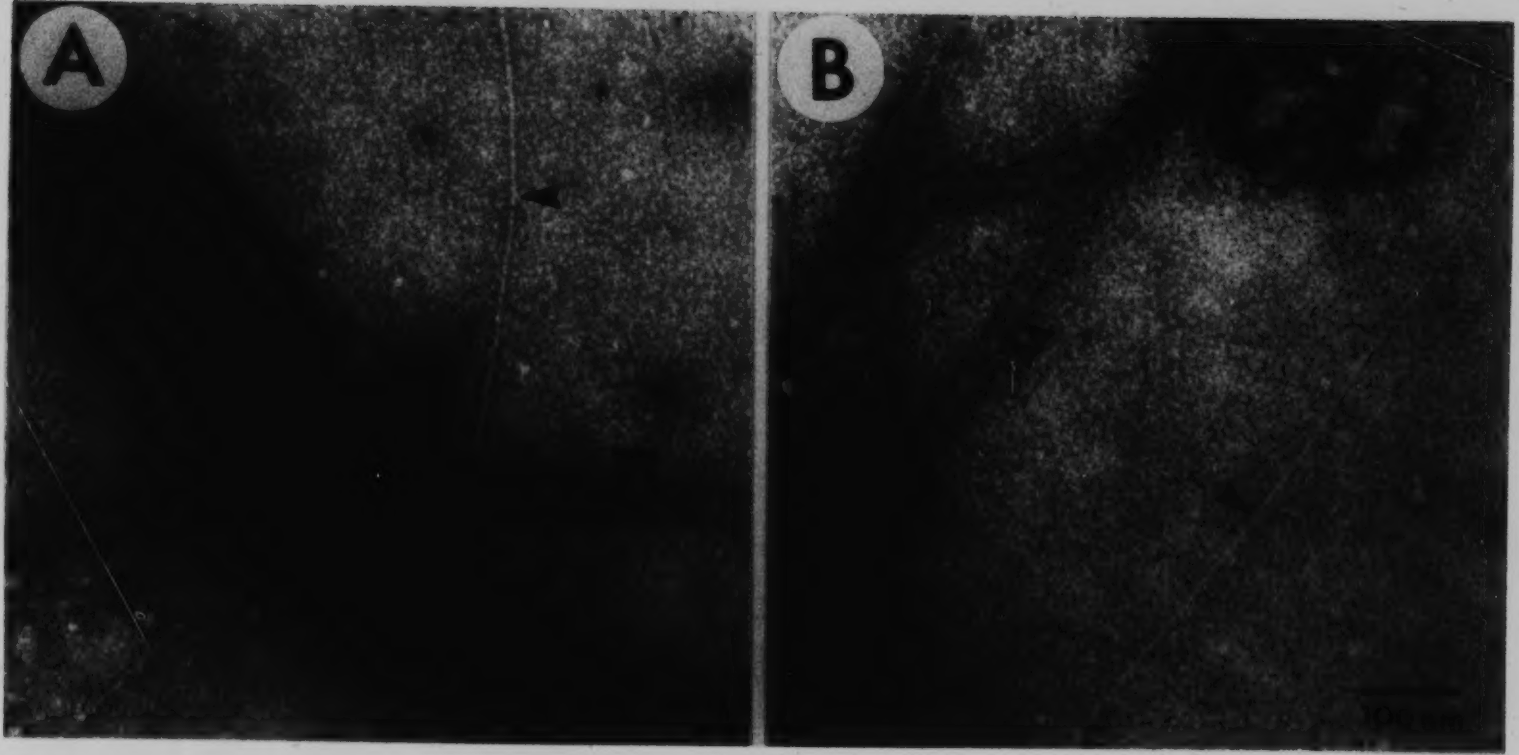
A
B
100 nm

FIGURE VII.2

Immunolabeling of the pili assembled by the pBP500$_9$ transconjugants of *P. aeruginosa* DB2. Micrographs of the pili produced by *P. aeruginosa* DB2 harboring pBP500$_9$ were taken under three different conditions: (A) Without the addition of anti-pili serum, (B) Incubated in the presence of anti-PAK pili serum, and (C) Incubated in the presence of anti-PAO pili serum.

A
B
C
100 nm

FIGURE VII.3

Electrophoresis and immunoblots of the pili purified from *P. aeruginosa* DB2 transconjugants. The pili, from the *P. aeruginosa* DB2 transconjugants harboring either pBP500, pBP540 or pBP500$_9$, were purified, fractionated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis and immunoblotted with either anti-PAK or -PAO pili serum. (A) Electrophoresis and immunoblots of transconjugants grown without antibiotic selection. (B) Immunoblots of pili purified from pBP500 transconjugants grown either in the absence (-Cm) or presence (+Cm) of chloramphenicol. The PAK and PAO pili were purified from *P. aeruginosa* strains 2PfS and DB2, respectively. The molecular weight standards are marked (in thousands) along the sides of the gel and the immunoblots.

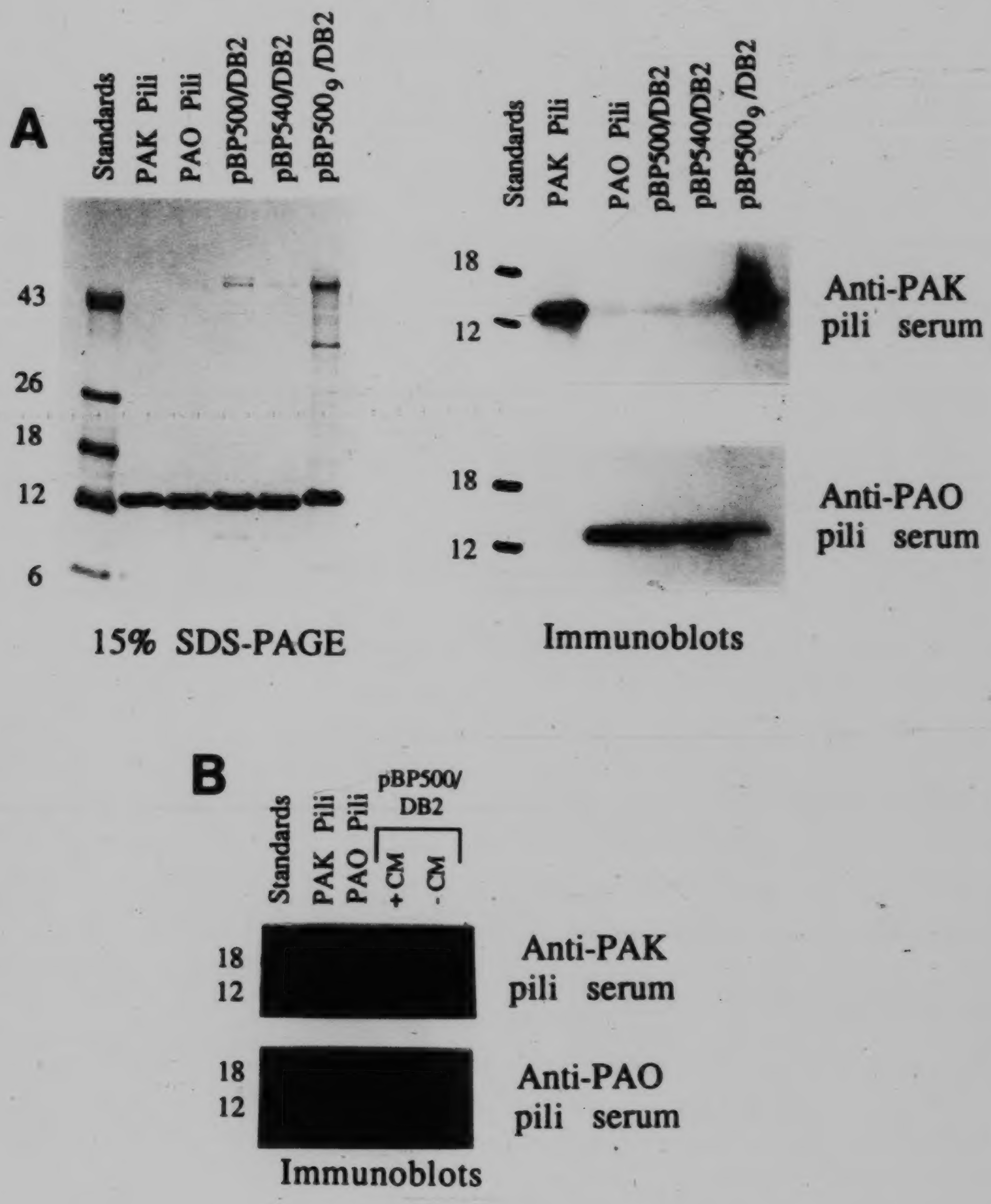
A
Standards
PAK Pili
PAO Pili
pBP500/DB2
pBP540/DB2
pBP500$_9$ /DB2
43
26
18
12
6
15% SDS-PAGE
Standards
PAK Pili
PAO Pili
pBP500/DB2
pBP540/DB2
pBP500$_9$ /DB2
18
12
Anti-PAK pili serum
18
12
Anti-PAO pili serum
Immunoblots
B
Standards
PAK Pili
PAO Pili
pBP500/ DB2
+CM
- CM
18
12
Anti-PAK pili serum
18
12
Anti-PAO pili serum
Immunoblots

TABLE VII.1

Strains and Plasmids.

Strain or plasmid	Relevant genotype or property	Source or reference
Strains		
Escherichia coli		
HB101	F^-, Sm^r, *rec*A13	1
CSH56	Nal^r	Cold Spring Harbor[a]
ED2602	*lac*$^-$ [F^+ *lac*$^+$]	Neil Willetts[b]
MM294 (pRK2013)	Mobilizing plasmid	13
BLP1	Tn*501* transposed into the HB101 genome	This study
P. aeruginosa		
2PfS	Multipiliated strain PAK	2
PAKp	Pilin-lacking mutant PAK	12
PAO1-leu	*leu*$^-$ mutant of PAO1 (ATCC 25247)	W. Paranchych
DB2	Multipiliated strain PAO1	14
BLP4	Tn*501*::*pil*A strain DB2	This study
Plasmids		
pLAFR	Cosmid cloning vector, Tc^r	6
Tn*501*::RSF1010	Broad-host-range plasmid, Hg^r	Dennis Ohman[c]
pBP310	PAK *pil*A clone in pLAFR	This study
pBP311	Tn*501*::*pil*A in pBP310	This study
pBP400	Control plasmid, chimera of pT7-4 and pKT210, Cb^rCm^r	12
pBP500	PAK pilin gene clone	12
pBP540	PAKΔ4 pilin gene clone	12
pBP580	PAKΔ8 pilin gene clone	12
pBP500_9	PAK Glu-5-to-Lys pilin gene clone	12

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TABLE VII.2

Relative Amounts of the PAK and PAO Pilins Detected in the Pili of the Multipiliated and Pilin-Lacking *P. aeruginosa* Transconjugants.

Plasmid (pilin phenotype)	Relative amounts of pilin types in *P. aeruginosa* host strain:[a]			
	DB2		BLP4	
	PAK	PAO	PAK	PAO
pBP400 (none)	-	+++	-	-
pBP500 (PAK)	+++	+	+++	-
pBP540 (PAKΔ4)	-	+++	-	-
pBP500$_9$ (PAK Glu-5-to-Lys)	+++	+	-	-

a. -, no pilin; +, minor pilin type; +++, major pilin type.

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Chapter VIII

THE ACTIVITY OF THE *PSEUDOMONAS AERUGINOSA* PILIN PROMOTER IS ENHANCED BY AN UPSTREAM REGULATORY SITE[1]

A. INTRODUCTION

In recent years, several genes from *Pseudomonas* strains have been found to utilize NtrA-dependent promoters. These genes include the *xylABC* operon of the TOL plasmid (Dixon, 1986), the carboxypeptidase G2 gene (Minton and Clarke, 1985) from *Pseudomonas putida*, and the chromosomally-encoded structural pilin gene, *pilA*, from *Pseudomonas aeruginosa* (Johnson et al., 1986). NtrA-dependent promoters have been characterized for many of the genes involved in nitrogen assimilation such as *glnA* in *Escherichia coli*, as well as the nitrogen fixation genes in several organisms including *Klebsiella pneumoniae* (Gussin et al., 1986). The *ntrA* gene product (NtrA), is an alternative, 54 kilodalton sigma factor which recognizes an atypical consensus sequence of CTGGYAYRN$_4$TTGCA, extending from position -26 to -10 relative to the transcription start point (*tsp*) (Gussin et al., 1986). The NtrA-dependent promoters in the enteric or nitrogen fixing organisms are regulated by the gene products of either *ntrC* (NtrC) or *nifA* (NifA) by binding to sequences upstream of theNtrA-dependent promoter. In *P. putida*, the *xylABC* operon is regulated by *xylR* and recent sequencing data has shown that the *xylR* gene product shares approximately 50% amino acid sequence identity with the NifA and NtrC regulatory proteins (Inouye et al., 1988), suggesting that Ntr-like promoters in *Pseudomonas* may be regulated in a manner similar to the NtrC and NifA controlled NtrA-dependent promoters.

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[1] A version of this chapter has been accepted for publication. **Pasloske**, B.L., Drummond, D.S., Frost, L.S. and Paranchych, W. 1989. Gene.

Johnson et al. (1986) recognized an NtrA-specific consensus sequence in the promoter for the *P. aeruginosa* pilin gene, although they did not demonstrate that it was dependent on an NtrA-like sigma factor. However, it was thought that this promoter, because of its similarity to an NtrA-like recognition site, might be regulated by a NifA- or NtrC-like protein. The DNA sequence upstream of *pilA* was searched for regions similar to NifA- or NtrC-binding sites. A putative regulatory sequence 115 base pairs (bp) upstream of the *tsp* was noted (Pasloske et al., 1988b). This sequence strongly resembles the NifA-binding sequence of *K. pneumoniae* which has the consensus sequence $TGTN_{10}ACA$ (Buck et al., 1986). To determine the functional relevance of this sequence, we compared the levels of pilin expression and quantitatively measured the promoter strengths of DNA fragments with and without the putative recognition sequence. The deletion of a 48-bp fragment containing this sequence resulted in much lower pilin and pilus expression and reduced the promoter activity by 90%. These results indicate that a region of DNA upstream of the Ntr-like pilin promoter is operating as a positive regulatory sequence affecting pilin gene expression.

B. MATERIALS and METHODS

1. Strains, Plasmids, and Media.

All of the bacterial strains and plasmids are listed in Table 1.

The media used were Luria-Bertani (LB) medium (Maniatis et al., 1985), and *Pseudomonas* isolation agar (Difco, Detroit, Michigan, USA). Antibiotic concentrations were as follows: mercuric chloride (Hg), 15 µg/ml; chloramphenicol and ampicillin, 50 µg/ml; carbenicillin, 300 µg/ml; tetracycline, 10 and 150 µg/ml, *E. coli* and *P. aeruginosa*, respectively.

2. Phage Sensitivity, Electron Microscopy, and Triparental Matings.

The phage sensitivity assays and the electron microscopy were performed as outlined in Chapter VII. Triparental matings were described in Chapter VI.

3. Recombinant DNA Techniques.

Restriction enzymes, T4 DNA ligase, and the large fragment (Klenow) of DNA polymerase I (PolIK) were purchased from Bethesda Research Laboratories, Gaithersburg, Md.. The restriction enzymes and T4 ligase were used according to the supplier's specifications.

Nick translations, DNA transfer to nitrocellulose filters, DNA filter hybridization, and colony filter hybridization have been described previously (Finlay et al., 1984). The transformation of

E. coli with plasmid DNA and the isolation of DNA fragments from 5% acrylamide were performed according to Maniatis et al. (1985). The isolation of small quantities of plasmid DNA was performed as described by Birnboim and Doly (1979).

To fill in the staggered ends created by an *Ava*I restriction digest, 3 µg of the digested DNA was resuspended in 20 1 of 5x ligase buffer, 3 µl of 10 mM dATP, 20 µl of a mixture with 3 mM each of dATP, dGTP, dCTP, and dTTP, and the total solution was brought to 100 µl with water. Five units of PolIK were added to the reaction mix, which was incubated 15 min at 37°C.

4. Construction of the Pilin Clones with and without *sipR*.

Fig. 1 presents the details of the cloning strategy. The *Msp*I$_{304-973}$ fragment of pBP001 was isolated from acrylamide and cloned into the *Acc*I site of pUC19 such that the transcription of *pil*A was in the direction opposite that of the beta-lactamase gene. This pilin clone (pDD201) was digested with *Ava*I, where restriction sites were located at position 352 and at the *Sma*I site within the multiple cloning site (MCS) of pUC19. The staggered ends were filled-in with PolIK, ligated, and then transformed into *E. coli* JM83 (pDD230). Finally, pDD201, pDD230, and pUC19 were digested with *Eco*RI in the presence of pKT210, ligated, transformed into *E. coli* JM83 and transformants selected on ampicillin-chloramphenicol-LB agar. The clones pDD210, pDD240, and pBP091 (pKT210 and pUC19 chimera) were selected on the basis that the orientation of the beta-lactamase gene was transcribed in the same direction as the gene coding for chloramphenicol resistance.

5. Construction of the Clones for Promoter Assessment.

To perform the promoter assays in *P. aeruginosa*, the pKO-4 chimeras and pKO-4 itself, were cloned into the *Eco*RI site of pKT210 as demonstrated in Fig. 2A for the construction of the control vector pBP600. In each of the clones the orientation of the pKO-4 chimera in pKT210 was such that the beta-lactamase gene was proximal to the gene coding for chloramphenicol resistance.

Most of the fragments which we wanted to assay did not have readily available restriction sites suitable for ligating into pKO-4. Therefore, these fragments were first cloned into the multiple cloning site of pUC18 or pUC19 which conferred *Eco*RI and *Hin*dIII restriction sites at each end of the fragments (Fig. 2B). These *Eco*RI-*Hin*dIII fragments were isolated from acrylamide, cloned into the *Eco*RI-*Hin*dIII sites of pKO-4 and then these constructs were cloned into pKT210 as shown in Fig. 2A. The *Alu*I$_{190-464}$ fragment was isolated from pBP001 (Fig. 3) and cloned into the *Sma*I site of pUC18 and pUC19, such that there were two different orientations of the *Alu*I fragment in the MCS. The *Eco*RI-*Sau*3A$_{352-485}$ fragment of pDD230 (Fig. 1), which includes the *Eco*RI site of the MCS, was cloned into the *Eco*RI-*Bam*HI sites of pUC18. The *Sau*3A$_{304-485}$ fragment of pDD201 (Fig. 1), which includes the *Bam*HI site of the MCS, was cloned in both orientations into the *Bam*HI site of pUC18. pUC linkers were not needed for the *Hin*dIII-*Sau*3A$_{1-485}$ fragment of pBP001 which was ligated into the *Hind*III-*Bam*HI sites of pKO-4 directly (Fig. 2B).

6. Construction of an Isogenic, Pilin-Lacking *P. aeruginosa* Strain.

pBP311, a cosmid clone containing a Tn*501* inactivated pilin gene from *P. aeruginosa* PAK, was mobilized from *E. coli* HB101 into *P. aeruginosa* PAK (*pil*$^+$) and transconjugants were selected on Hg-*Pseudomonas* isolation agar. Many of the isolates were assayed for their sensitivity to PO4 and approximately 30% were phage-resistant. These same isolates were tetracycline-sensitive, indicating loss of the cosmid DNA. Direct examination of cells from one of these colonies (*P. aeruginosa* BLP2) by electron microscopy revealed an apparent absence of pili. Also, the pilin subunit was not detected in a crude membrane extract upon immunoblotting with anti-PAK pili serum. Finally, evidence that homologous recombination had occurred between the Tn*501*-containing pilin gene and the chromosome was provided by a comparison of the restriction patterns of the mutant and wild-type pilin genes. The mutant pilin gene produced the fragment sizes expected of a chromosomal DNA having undergone the anticipated gene replacement (data not shown).

7. Membrane Purification.

The *P. aeruginosa* BLP2 transconjugants were grown to 1x10^9 CFU/ml in 500 ml of chloramphenicol-LB broth at 30°C with shaking. The crude membrane was purified according to the procedure of Hancock and Nikaido (1978) with some modifications. The cells were harvested, resuspended in 3 ml of 20% (wt/vol) sucrose-10mM Tris hydrochloride (pH 8.0), 0.1 mg of deoxyribonuclease was added and the cells incubated 20 min at room temperature. The cells were frozen, followed by thawing at room temperature and two passages through a French pressure cell at 15,000 lb/in^2 at 4°C. The membranes were incubated on ice for 10 min after the addition of 0.5 mg of lysozyme. Spheroplasts and cell debris were pelleted by centrifugation for 10 min at 12,000 x *g*. The supernatant was layered upon a sucrose gradient composed of 4 ml of 18% (wt/vol) sucrose-10 mM Tris hydrochloride (pH 8.0) layered onto 2 ml of 70% (wt/vol) sucrose-10 mM Tris hydrochloride (pH 8.0). A buffer of 10 mM Tris hydrochloride (pH 8.0) was used to raise the level of the membrane fraction to the top of the centrifuge tube. The gradients were spun for 2 h at 38,000 rpm using an SW41 rotor. The crude membrane at the 18%:70% sucrose interface was collected with a pasteur pipette and diluted with 10 mM Tris hydrochloride (pH 8.0) to bring the volume to the top of the centrifuge tube. The crude membrane was pelleted by another 2 h centrifugation at 38,000 rpm with an SW41 rotor and it was resuspended in 200 µl of 10 mM Tris hydrochloride (pH 8.0).

C. RESULTS and DISCUSSION

1. Pilin and Pili Expression is Enhanced by *sip*R.

The plasmid pBP001 (Pasloske et al., 1985) contains the *P. aeruginosa* pilin gene from the prototrophic strain PAK. Derivatives of pBP001 were constructed with and without the sequence resembling a NifA-recognition site located between positions 309 to 325 (Fig. 3). These derivatives containing *pilA* plus either 72 (pDD240) or 119 (pDD210) bp upstream of the transcription start point (*tsp*) were either *sipR*$^-$ or *sipR*$^+$, respectively (Fig. 1). *sipR* is defined as the DNA fragment between restriction sites *Msp*I$_{304}$ and *Ava*I$_{352}$. These constructs and the control vector, pBP091 (Fig. 1), were mobilized by a triparental mating into *P. aeruginosa* BLP2, an isogenic, *pil*$^-$ mutant of *P. aeruginosa*. Examination of these transconjugants by electron microscopy showed that the *sipR*$^+$ cells produced about 3 pili per cell while no pili were observed extending from the surface of the *sipR*$^-$ cells or the control pBP091 transconjugants, although some pili were seen in the background detached from the *sipR*$^-$ cells. The *sipR*$^+$ cells were sensitive to the pilus-specific bacteriophage PO4, indicating the presence of retractile pili but the cells harboring the control vector or the *sipR*$^-$ pilin clone were resistant to the phage.

To assess the effect of *sipR* on the expression of membrane-associated pilin, crude membrane extracts were prepared from transconjugants containing each of the three plasmids. In Fig. 4, a Coomassie blue stained gel of the membranes fractionated by SDS-PAGE reveals that the *sipR*$^+$ clone produced a band with the mobility of mature pilin whereas no pilin was observed for the control vector or *sipR*$^-$ extracts. However, an immunoblot of the same gel with anti-PAK pili serum detected some pilin in the membranes of the *sipR*$^-$ clone. There was no detectable pilin in the control membranes. These data indicate that the deletion of *sipR* decreases pilin expression but does not totally abolish it.

2. *sipR* Increases the Pilin Promoter Activity

Several different DNA fragments upstream of the pilin gene were cloned into the promoter assessment vector pKO-4 upstream from the reporter gene *galK* (Fig. 2). We were not able to detect any galactokinase activity from *P. aeruginosa* PAO1 unless a cloned *galK* gene was provided. Therefore, it was not necessary to construct a *galK*$^-$ mutant for the promoter experiments. Measurements in *P. aeruginosa* were made possible by cloning the pKO-4 chimeras into the broad host range vector pKT210 (Fig. 2). Fig. 5 depicts the fragments cloned into pKO-4 and the levels of galactokinase activity associated with each of them in *P. aeruginosa*.

The control vector, pBP600, had lower galactokinase activity than pBP094 which contained only the pilin promoter (nucleotides 397 to 413, Fig. 3). If both *sipR* and the pilin promoter were included in the same fragment (pBP057), the promoter activity increased 13-fold. Extending the fragment in pBP057 to position 190 (pBP083) did not result in a noticeable difference in promoter activity.

If the fragment cloned into pBP057 was ligated in the opposite orientation (pBP058), the promoter activity was less than that of the control vector, pBP600. These data demonstrate that transcription is unidirectional for a DNA fragment containing *sipR* and the pilin promoter. However, if the cloned fragment extended to position 190 (pBP085), a weak but significant level of promoter activity was detected. This result suggested that a promoter was located between *Alu*I$_{190}$ and *Msp*I$_{304}$ and indeed, a sequence similar to an Ntr-promoter was located between positions 266 and 230 (Fig. 3). The direction of transcription would be opposite to that of the pilin promoter.

It is interesting to speculate that *sipR* might be part of two divergent promoters, regulating the transcription of both the pilin promoter and the putative upstream NtrA-dependent promoter. Presently, we cannot assign a purpose to this promoter. There is one open reading frame immediately downstream of this putative promoter, starting at position 230 (Fig. 3), but it has a very poor ribosome binding site such that the probability of a translated product is low.

Consistent with the divergent promoter theory, in *Caulobacter crescentus*, the positive control region element II-1 is located between two NtrA-dependent, divergent promoters of transcription units I and II of the *fla* gene cluster (Mullin et al., 1987). Similar to the sequence resembling a NifA-recognition site within *sipR*, the II-1 regulatory element is centered approximately 100 nucleotides upstream of the *tsp*'s of transcription units I and II. The sequence of element II-1 is very similar to *sipR* but it lacks the key nucleotides which constitute a NifA-binding sequence (Fig. 6). There are 21 of 29 (~72%) nucleotides of element II-1 identical to the *sipR* sequence.

The plasmid pBP610, which has both the pilin promoter and *sipR*, produced very high levels of galactokinase in *P. aeruginosa* compared to the control vector pBP600. pBP610 is the pKT210 derivative of pBP010 (Fig. 2B). pBP010 was previously tested for galactokinase activity in *E. coli* and its promoter strength was just above background levels (Finlay et al., 1986) suggesting that there are transcriptional factors regulating *pilA* unique to *Pseudomonas* which are not found in *E. coli*.

pBP610 consistently produced higher levels of galactokinase than pBP057 and pBP083 suggesting that there was another DNA element which was enhancing transcription between *Hin*dIII$_1$ and *Alu*I$_{190}$. This situation may be analogous to the upstream region of *glnA* in *E. coli* which has five distinct binding sites for NtrC. Promoter activation by NtrC may be dependent upon the quality and number of NtrC binding sites (Reitzer and Magasanik, 1986). Similarily, there may be another *sipR* sequence between *Hin*dIII$_1$ and *Alu*I$_{190}$ (Fig. 1) which may have an additive effect upon pilin promoter strength.

3. Conclusions.

(1) A DNA sequence (*sipR*) located between -72 and -119 relative to the *tsp* of the pilin gene from *P. aeruginosa*, enhanced transcription of the pilin gene by more than 10-fold.

(2) *sipR* may be part of a divergent regulatory system, controlling the expression of two opposing Ntr-like promoters.

FIGURE VIII.1

Construction of the pilin clones. For a more detailed explanation refer to *Materials and Methods*, part 6. The numbering beneath the fragments refer to the nucleotide positions in pBP001 (Fig. 1; Pasloske et al., 1985). The thin lines indicate the fragments cloned from pBP001. The open boxes represent the Ntr-pilin promoter and the closed box signifies the region resembling a NifA-recognition sequence. The thick lines of pDD201 and pDD230 signify the DNA encoded by pUC19. Abbreviations: *Alu*I, A; *Ava*I, V; *Bam*HI, B; beta-lactamase, ß-lac; chloramphenicol resistance gene, Cm; *Eco*RI, E; *Hind*III, H; *Msp*I, M; *Sau*3A, U; *Sma*I, S.

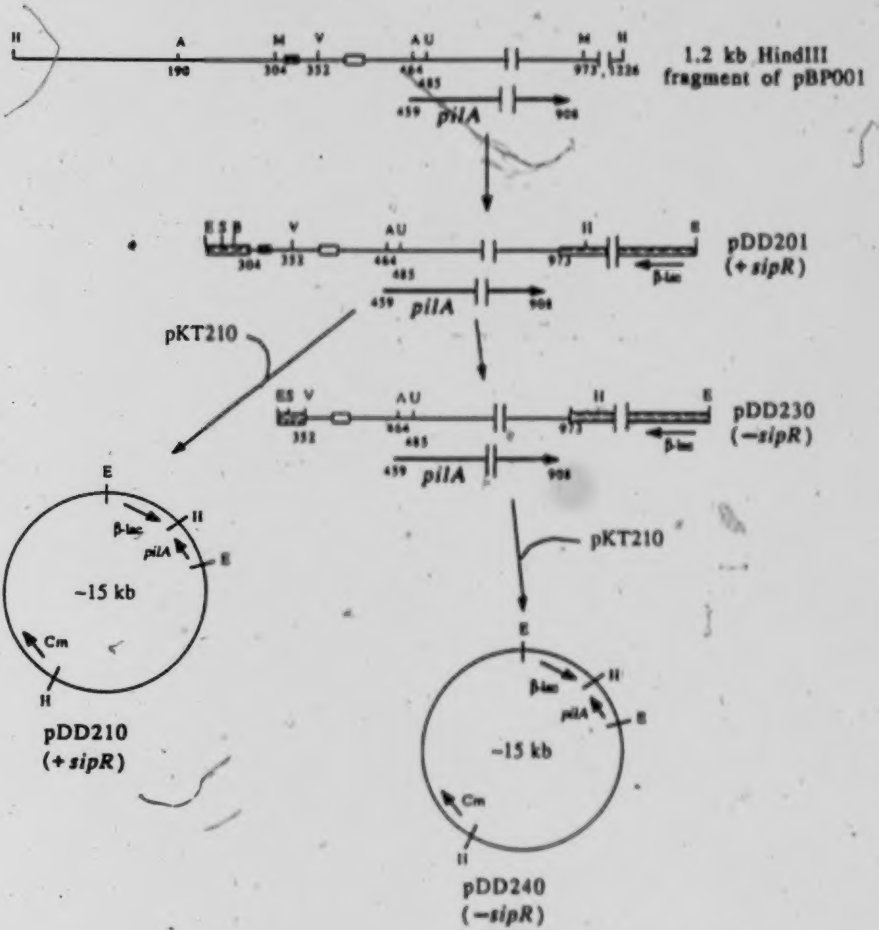

H
A
190
M
304
V
352
A U
464
485
M
973
H
1226
1.2 kb HindIII fragment of pBP001
459
pilA
908
E S B
V
304
352
A U
464
485
H
973
E
β-lac
pDD201
(+sipR)
459
pilA
908
pKT210
E S V
352
A U
464
485
H
973
E
β-lac
pDD230
(−sipR)
459
pilA
908
E
H
β-lac
pilA
E
~15 kb
Cm
H
pDD210
(+sipR)
pKT210
E
H
β-lac
pilA
E
~15 kb
Cm
H
pDD240
(−sipR)

FIGURE VIII.2

Construction of the clones for promoter assessment. For a more detailed explanation refer to *Materials and Methods*, part 7. (A) Strategy for the construction of the pKT210 derivatives. (B) Strategy for constructing pKO-4 clones and their resultant pKT210 chimeras. The open boxes indicate fragments taken from pBP001 and the thin lines represent the MCS of the pUC vectors. Abbreviations: *AvaI*, V; *BamHI*, B; beta-lactamase, ß-lac; chloramphenicol resistance gene, Cm; *Eco*RI, E; galactokinase K, *galK*; *Hin*dIII, H; *Sau*3A, U. The vector pKO-4 used in these experiments is a derivative of pKO-1 (McKenney et al., 1981).

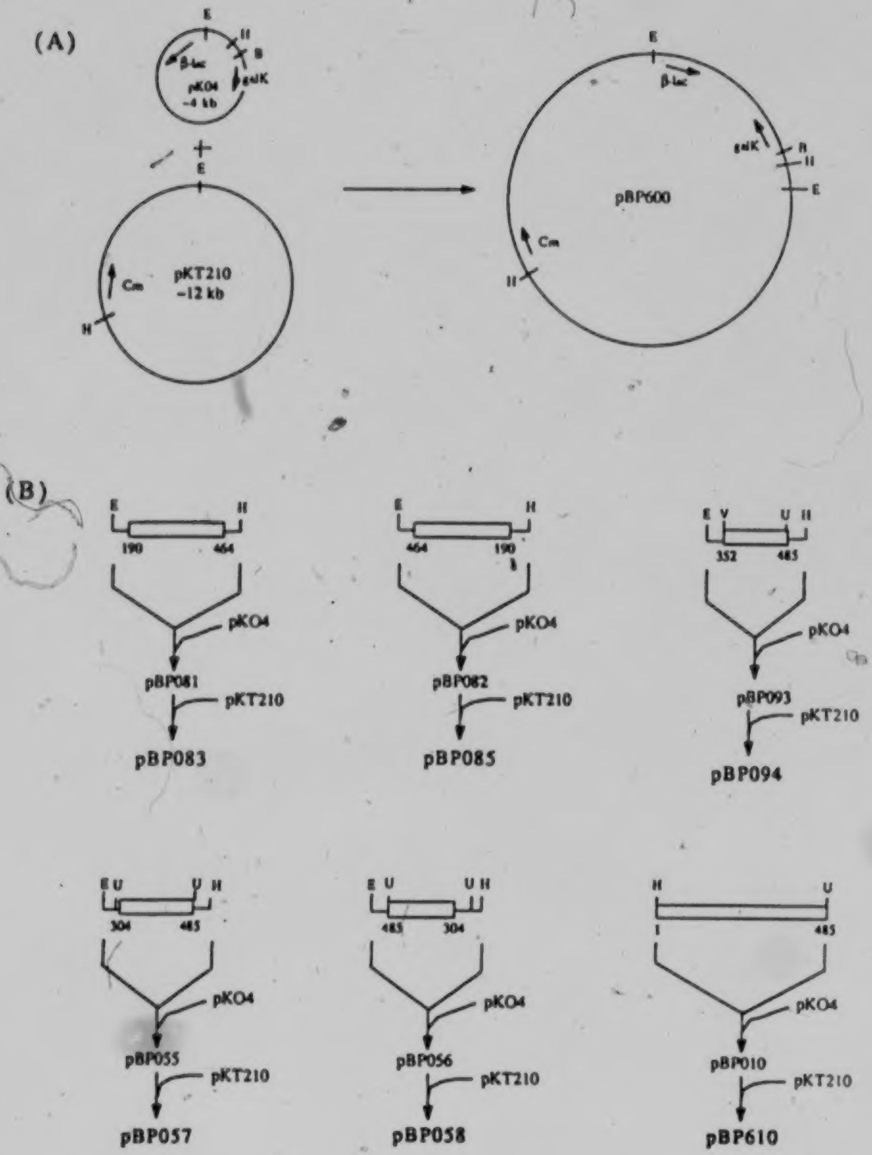

(A)
E
H
B
β-lac
galK
pKO4
~4 kb
+
E
Cm
pKT210
~12 kb
H
E
β-lac
galK
B
H
E
pBP600
Cm
H
(B)
E
H
190
464
pKO4
pBP081
pKT210
pBP083
E
H
464
190
pKO4
pBP082
pKT210
pBP085
E
V
U
H
352
485
pKO4
pBP093
pKT210
pBP094
E
U
U
H
304
485
pKO4
pBP055
pKT210
pBP057
E
U
U
H
485
304
pKO4
pBP056
pKT210
pBP058
H
U
1
485
pKO4
pBP010
pKT210
pBP610

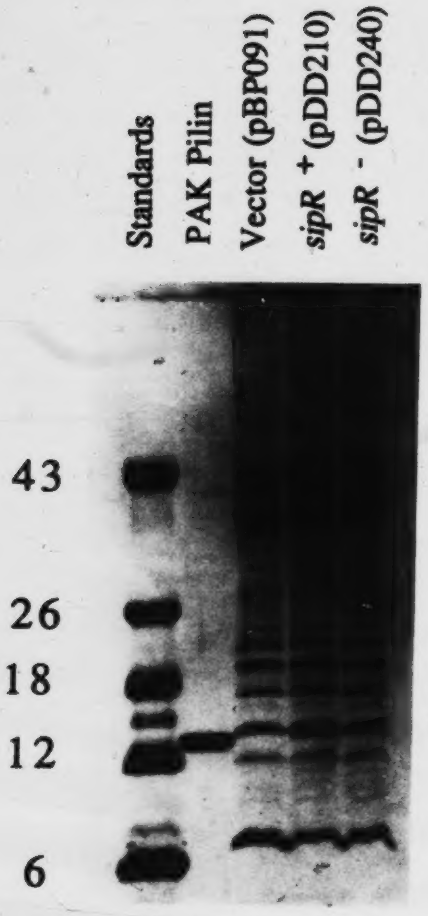
FIGURE VIII.3

The first 521 nucleotides of pBP001 and their relevant features. The nucleotide positions are marked above the DNA sequence. The pilin coding sequence begins at 459 and the encoded amino acids are printed beneath the sequence. Sequences homologous to the NifA- and NtrA-recognition consensus sequences (Buck et al., 1986; Gussin et al., 1986) are indicated above or below the sequence depending on the direction of transcription. The *sipR* region is designated by a broad, stipled underline. The horizontal arrows indicate the direction of transcription and of a putative open reading frame (ORF). The *tsp* is indicated by a vertical arrow as mapped by Johnson et al. (1986). Abbreviations: R, purine; Y, pyrimidine.

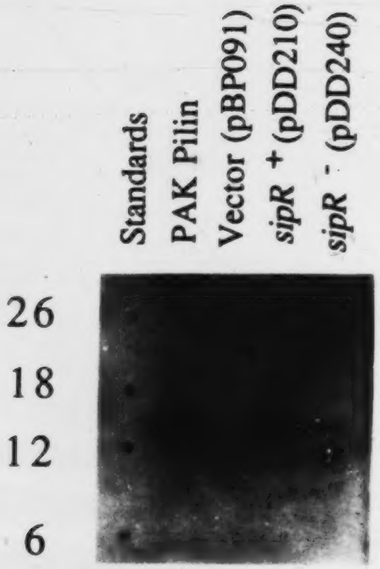
```
                           25                                 50                              75
AAGCTTTCCCTGTCCAGGCTGTTCAGGTCGCAATAGGCGATGCCGAACTGCTCGGCGGACAATTCGGCCAGGGCC
GGCGAAAGGGACAGGTCCGACAAGTCCAGCGTTATCCGCTACGGCTTGACGAGCCGCCTGTTAAGCCGGTCCCGG


                          100                                125                             150
AGGCCGCTTACCAGCTTGTTCTGCACCAGGTGCGTCACCAGCGACAGCTTGTTGCGCTGCGCCTGCGTCTGGGCC
TCCGGCGAATGGTCGAACAAGACGTGGTCCACGCAGTGGTCGCTGTCGAACAACGCGACGCGGACGCACACCCGG


                          175                                200                              225
TGCAGGGGCGGTCTTTTCGTCGAGTAGATTGGCTTGGACGAGCTGTCGGGACAGACCGCTCAGTTGGATGCTGTCG
ACGTCCCCGCCAGAAAAGCAGCTCATCTAACCGAACCTGCTCGACAGCCCTGTCTGGCGAGTCAACCTACGACAGC

                          250                                275                             300
TTCATGGAGAAGGAAATCGCAGAGGGCTATTGAAGTGCCTTATAACGCACATAACAGGGTCTGCCAAATCGGGGG
AAGTACCTCTTCCTTTAGCGTCTCCCGATAACTTCACGGAATATTGCGTCTATTGTCCCAGACGGTTTAGCCCCC
←————┘                        || |  .    |||||
  (Putative ORF)          ←— ACGTT----RYAYGGTC (Consensus NtrA Promoter)

       TGT-----------ACA (Consensus NifA Recognition Sequence)
  MspI |||      320   |||                        350  AvaI                        375
AGTCCGGCTGTCAAAAAGTGTCACATCCTGTCCGTTTTAAGTTTGAGTCTCCTCGGGAAGGGCATAAGACGGCCT
TCAGGCCGACAGTTTTTCACAGTGTAGGACAGGCAAAATTCAAACTCAGAGGAGCCCTTCCCGTATTCTGCCGGA

                        sipR

(Consensus NtrA Promoter) CTGGYAYR----TTGCA ——→
                  395     |||||||     |||              425                            450
TGCTCGCTATGTGAACGGTATTTGGCATGGTAAGTGCTTGGTAGGGTTAGGCGTTAGGCCTATACATATCAATGG
ACGAGCGATACACTTGCCATAAACCGTACCATTCACGAACCATCCCAATCCGCAATCCGGATATGTATAGTTACC
                                                 ↑+1 (Transcription Start Point)
                        475                              500                       520
AGATATTCATGAAAGCTCAAAAAGGCTTTACCTTGATCGAACTGATGATCGTGGTTGCGATCATCGGTATC
TCTATAAG
        MetLysAlaGlnLysGlyPheThrLeuIleGluLeuMetIleValValAlaIleIleGlyIle
        pilA
```

FIGURE VIII.4

Levels of membrane-associated pilin in *sipR*$^+$ and *sipR*$^-$ clones. The crude membrane extracts were fractionated on 15% sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970) and stained with Coomassie blue or transferred to nitrocellulose and immunoblotted with anti-PAK pili serum (Pasloske et al., 1988a). The quantities of PAK pilin standard used were 3 μg for Coomassie blue staining and 0.5 μg for immunoblotting. The molecular weight standards ($x10^{-3}$) are printed along the sides of the gel and immunoblot.


Standards
PAK Pilin
Vector (pBP091)
sipR + (pDD210)
sipR - (pDD240)
43
26
18
12
6

15% SDS-PAGE


Standards
PAK Pilin
Vector (pBP091)
sipR + (pDD210)
sipR - (pDD240)
26
18
12
6

IMMUNOBLOT

FIGURE VIII.5

Relative promoter strengths from DNA fragments upstream of the PAK pilin gene. The fragments assessed are aligned above the restriction sites of pBP001 from which they were derived. The arrows indicate the orientation in which they were cloned into pKO-4. A unit of galactokinase activity is defined as nanomoles of galactose phosphorylated per minute per $8x10^8$ CFU. The transconjugants of *P. aeruginosa* PAO1 were grown to $8x10^8$ CFU/ml at 37°C in chloramphenicol-LB broth with shaking and then the galactokinase K assay was performed as described by McKenney et al. (1981) except that the specific activity of the undiluted $[^{14}C]$galactose was 59.6 mCi/mmol and the assays were performed at 37°C rather than 32°C. Similar results were obtained in *P. aeruginosa* PAK as well. The data presented are representative from one of three experiments involving galactokinase assays. The standard deviations from three separate experiments for each clone was approximately ±10% of the means.

DNA Fragment Cloned into GalK Expression Vector	Units of Galactokinase Produced	Per Cent Activity Relative to pBP610 Promoter Activity
pBP600 (vector alone)	3	4
→ pBP094	5	6
→ pBP057	66	86
← pBP058	0.7	0
→ pBP083	65	84
← pBP085	13	17
→ pBP610	77	100

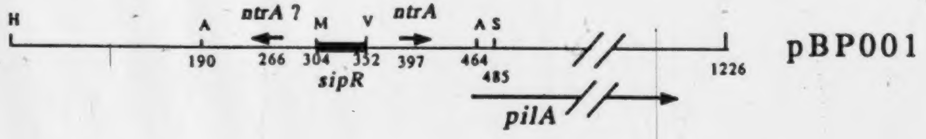

H
A
ntrA ?
M
V
ntrA
A S
190
266
304
332
397
464
485
1226
sipR
pilA
pBP001

FIGURE VIII.6

DNA sequence comparison between the *sipR* sequence and element II-1 of transcription units I and II from the *fla* gene cluster of *Caulobacter crescentus* (Mullin et al., 1987). The vertical lines indicate nucleotide identities. The numbering is relative to the transcription start points of *pilA* and transcription unit II. The underlined residues in the *sipR* sequence distinguish the nucleotides homologous to the NifA-binding sequence (Gussin et al., 1986).

```
                           -100
sipR              TGTCAAAAAGTGTCACATCCTGTCCGTTTTAA
                   | ||||||| | | || || || || |||
Element II-1      CGGCAAAAAGCGCCGCACCCGGTGCGATTTTT
                            -100
```

TABLE VIII.1

Strains and Plasmids.

Strain or plasmid	Relevant genotype or property	Source or reference
Strains		
Escherichia coli		
HB101	*recA*	Boyer and Roulland-Dussoix, 1969
JM83	Δ(*lac-proAB*), *lacZ*ΔM15	Vieira and Messing, 1982
P. aeruginosa		
PAO1	Prototroph	ATCC 25247
PAK	Prototroph	ATCC 25102
BLP2	Tn*501*::*pilA* strain PAK, Hg^r	This study
Plasmids		
pBP311	Tn*501*::*pilA* in pBP310	Chapter VII
pKT210	Broad host range vector, Cm^r	Bagdasarian et al. 1981
pUC18	MCS, Ap^r	Yanisch-Perron et al., 1985
pUC19	MCS, Ap^r	Yanisch-Perron et al., 1985
pKO-4	Promoter assessment vector, Ap^r see Fig. 4A	McKenney et al., 1981
pBP001	1.2-kb *Hind*III fragment cloned in pUC8 containing the PAK *pilA*	Pasloske et al., 1985
pDD210	*pilA* with *sipR*	This study
pDD240	*pilA* without *sipR*	" "
pBP091	control vector	" "
pBP600	see Fig. 4A	" "
pBP610	see Fig. 4B	" "
pBP057	see Fig. 4B	" "
pBP058	" "	" "
pBP083	" "	" "
pBP085	" "	" "
pBP094	" "	" "

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Chapter IX

THE SURFACE MAPPING OF THE NMEPHE PILI OF *PSEUDOMONAS AERUGINOSA* USING PILUS-SPECIFIC PHAGE: THE C-TERMINAL DISULFIDE LOOP IS EXPOSED AT THE PILUS TIP[1]

A. INTRODUCTION

The pili of *P. aeruginosa* act as receptors for a number of pilus-specific bacteriophage. The bacteriophage PO4, f116, and B9 have noncontractile tails and the pilus receptors are laterally located while the receptor for the filamentous bacteriophage Pf is located at the tip of the pilus (3, 4, 18). Chemical mutagenesis was used to generate three different pilin gene mutants from *P. aeruginosa* PAK which altered the susceptibility of *P. aeruginosa* to the pilus-specific phage. DNA sequencing of the pilin genes positioned these missense mutations within epitopes which were previously mapped as major antigenic domains suggesting that these substitutions were at the pilus surface.

As well, five different pili prototypes were expressed in two isogenic, pilin-lacking *P. aeruginosa* strains and the phage sensitivity of each pili type was assayed. Collectively, data from these experiments, phage sensitivity profiles from the mutant pili studies, and observations from previous immunological studies provide compelling evidence that the C-terminal disulfide loop of

[1] A version of this chapter has been submitted for publication. **Pasloske, B.L., and Paranchych, W.**

P. aeruginosa PAK pilin is a domain of the pilus tip.

B. MATERIALS and METHODS

1. Strains, Plasmids, Growth Media, and Antibiotic Concentrations.

All of the bacterial strains, bacteriophage and plasmids are listed in Table 1.

The media used were Luria-Bertani (LB) medium (10), and *Pseudomonas* isolation agar (Difco, Detroit, Michigan, USA). The antibiotic concentrations were as follows: mercuric chloride (Hg), 15 μg ml^{-1}; ampicillin, chloramphenicol, and kanamycin, 50 μg ml^{-1}; carbenicillin, 100 μg ml^{-1}, *P. aeruginosa* strains PAK and BLP2, and 300 μg ml^{-1}, *P. aeruginosa* strains PAO1-leu and BLP3; tetracycline, 150 μg ml^{-1}. When the cells were grown in LB broth, the antibiotic concentrations were half of the above concentrations.

2. The Spot Test for Bacteriophage Lytic Activity.

The *P. aeruginosa* strains harboring the pilin plasmid constructs were grown at 30°C with shaking in the presence of carbenicillin to approximately 5 x 10^8 colony forming units ml^{-1}. Aliquots of 0.1 ml of each strain were pipetted into 2 ml of 0.7% soft agar, vortexed well and poured onto carbenicillin-LB agar plates. Serial, ten-fold dilutions of each bacteriophage were made in LB broth and 2 μl of each dilution was pipetted on the double-agar layer plate. The cells were incubated overnight at

30°C and local clearing indicated phage susceptibility.

3. Preparation of Bacteriophage.

Confluent lysis of bacterial growth on standard double-agar-layer plates at 37°C were used to obtain high titre phage stocks of PO4, B9, and f116. The phage were extracted with LB broth for 3 h, followed by centrifugation to remove debris (3). *P. aeruginosa* PAO1-leu was the host bacteria for f116 and strain PAK was used for PO4 and B9. The concentrated titres of each of these bacteriophage were 1x10^{9} plaque forming units ml^{-1}.

P. aeruginosa PAK was infected with bacteriophage Pf at a multiplicity of infection of 0.01 in LB broth and grown overnight at 37°C with shaking at 250 rpm. Centrifugation at 7,000 x *g* for 15 min was used to remove cell debris (4). The concentrated titre of Pf was 1x10^{10} plaque forming units ml^{-1}.

4. Plasmid Transfer by Conjugation.

Triparental matings were performed as described earlier in Chapter VI.

5. Electron Microscopy.

The methods used for electron microscopy have been cited earlier in Chapter VII.

6. Nitrosoguanidine Mutagenesis of the PAK Pilin Gene.

Chemical mutagenesis of the PAK pilin gene in pBP500 was performed as described previously in Chapter VI.

7. Recombinant DNA Techniques and DNA Sequencing.

The restriction enzymes and T4 DNA ligase were purchased from Bethesda Research Laboratories, Gaithersburg, MD. The Sequenase DNA sequencing kit was purchased from Sequenase United States Biochemical Corporation, Cleveland, Ohio. These products were used according to the suppliers specifications.

Maniatis *et al.* (10) describes the procedures for the transformation of *E. coli* with plasmid DNA and the isolation of DNA fragments from 5% acrylamide. The isolation of plasmid DNA by the rapid alkaline method was performed as described by Birnboim and Doly (2). DNA primers used to sequence the mutant pilin genes were from positions 426 to 440 (5'dGCGTTAGGCCTATAC3'), and 942 to 928 (5'GATAGTTCAACACCA3') of pBP001 (14). They were purchased from Regional DNA Synthesis Laboratory, Calgary, Alberta, Canada.

8. Cloning the Five Prototype Pilin Genes into a Broad Host Range Vector.

The construction of the pilin clones was similar to the design of pBP500 (16). The pilin genes from the strains PAK, PAO, K122-4, CD, and RMCK1 are all centered within a 1.2-kilobase *Hin*dIII fragment (14, 15, 17, 21). The pilin gene of strain RMCK1 is almost identical to the pilin gene of PAK with the exception of two nucleotide changes. The codon Ile-98 of RMCK1 was ATT, resulting in a silent mutation while the C-terminal codon AAG of the RMCK1 pilin gene coded for lysine instead of arginine as found in the PAK pilin gene (data not shown). These fragments were each cloned into the *Hin*dIII site of pUC19 such that both the pilin gene (*pilA*) and the gene encoding ß-lactamase (ß-lac) were transcribed in the same direction (Fig. 1). These constructs were digested with *Eco*RI and ligated into the EcoRI site of pKT210 such that ß-lac was proximal to the gene encoding chloramphenicol resistance.

Construction of the clone containing the P1 pilin gene was slightly different. The clone pP1-001 contains a 3.5-kilobase *Hin*dIII-*Bam*HI fragment with the P1 pilin gene in pUC19 (17). pP1-001 was digested with *Eco*RI and ligated into the *Eco*RI site of pKT210 such that *pilA* was proximal to the chloramphenicol resistance gene (Fig. 1).

The control vector pBP090, is a chimera of pUC19 ligated into the *Eco*RI site of pKT210 with the same orientation as in the five *Hin*dIII pilin gene clones.

9. Construction of the Pilin-Lacking, Isogenic *P. aeruginosa* PA01-leu.

A triparental mating was performed to mobilize pBP311, a cosmid containing a Tn*501* disrupted pilin gene from *P. aeruginosa* PAK, into *P. aeruginosa* PA01-leu and transconjugants were selected on Hg-tetracycline-*Pseudomonas* isolation agar. One of the colonies was grown at 37^{o}C in LB broth with shaking, and passaged every 12 h for 120 h without antibiotic selection to allow gene replacement to occur through a double homologous recombination. A successful gene replacement would result in *P. aeruginosa* becoming resistant to the pilus-specific bacteriophage PO4. Therefore, to select for *pilA*$^-$ mutants, the passaged cells were incubated with PO4, at a multiplicity of infection of 10,000, for 30 min at 37°C and then grown at 30°C on Hg-LB agar. Approximately 0.5% of these cells were phage resistant and tetracycline resistant indicating that a possible gene replacement had occurred, although pBP311 remained in the cells.

To cure the phage resistant mutant of pBP311, the temperature sensitive plasmid pMR5 was mobilized into the resistant strain, and transconjugants selected on carbenicillin-*Pseudomonas* isolation agar. Both pMR5 and pBP311 belong to the same incompatability group, IncP, and therefore cannot co-exist in *P. aeruginosa*.

Having expelled pBP311 with pMR5 from the phage resistant strain, the bacteria were cured of pMR5 by growing the cells at 44°C for 18 h, the plasmid being unstable at this temperature. Loss of pMR5 was assayed by sensitivity to carbenicillin and tetracycline. One isolate (*P. aeruginosa* BLP3) which had lost pMR5, maintained its resistance to Hg and PO4 phage. Equivalent digests of genomic DNA from the mutant BLP3 and the wild-type strain PAO1-leu, which were probed with a DNA pilin probe, were found to have different restriction patterns (data not shown). The mutant had the restriction pattern expected from a Tn*501* insertion into the pilin gene. Electron microscopy showed that these cells were unpiliated. As well, mobilizing pBP165 (PAO pilin clone) into the mutant restored PO4 sensitivity and pili were assembled as observed by electron microscopy.

C. RESULTS

1. Selection, Sequencing, and Phenotypic Expression of the Mutant PAK Pilin Genes.

Recently, chemical mutagenesis of the PAK pilin gene was used to isolate a mutant pilin gene which expressed a pilin protein conferring resistance to the pilus-specific bacteriophage PO4. The mutation was a glutamate to lysine substitution at position 5 of the mature pilin (16). Phage resistance resulted because the mutant pilin was incapable of polymerizing into pili (Chapter VII). The selection procedure was modified to identify mutant pilins which conferred resistance to PO4 but also maintained their assembly capabilities. All of the mutants resistant to PO4 were also tested for sensitivity to the pilus-specific phage B9. A PO4-resistant, B9-sensitive phenotype indicated the presence of assembled PAK pili.

Twelve PO4 resistant strains were collected on this basis. The mutated pBP500 plasmids were isolated from *P. aeruginosa* PAKp and transformed into *E. coli* DH5α. Plasmid DNA was purified, the 1.1-kilobase *Hin*dIII-*Eco*RI fragment carrying the pilin gene was isolated from acrylamide, cloned into M13mp18 and M13mp19, and sequenced using synthetic primers which were made to anneal just outside either end of the PAK pilin gene. Of the initial 12 mutants selected, only three different point mutations were detected (Table 2). The three mutant clones and the wild-type pBP500 were mobilized into the pilin-lacking strains of *P. aeruginosa* PAK and PA01-leu, strains BLP2 and BLP3, respectively.

Using the double-agar-layer plate method the eight different strains were tested for their sensitivity to four different pilus-specific bacteriophages: PO4, B9, Pf and f116. Increments or degrees of sensitivity to the phage were detected by making serial, 1:10 dilutions and observing the lowest titre at which complete lysis was observed. The most pronounced phenotype was produced by the Gly-113-to-Glu pilin, conferring total resistance to PO4 in both BLP2 and BLP3 (Table 3). The sensitivities of this pilin mutant to other phage were identical to the wild-type strains except that there was a distinct 10-fold increase in sensitivity to Pf in BLP2 (i.e., complete lysis was observed at a concentration of 1×10^5 PFU ml^{-1} versus 1×10^6 PFU ml^{-1} with wild-type pili).

In the pilin-lacking PAO1-leu host, the Pro-72-to-Leu and the Ser-131-to-Asn pilins conferred sensitivity profiles no different than that of the wild-type pilin. In contrast, in a pilin-lacking PAK background, the Ser-131-to-Asn mutation abolished Pf sensitivity but maintained sensitivity towards PO4, B9 and f116. The Pro-72-to-Leu pilin conferred slightly greater resistance to PO4, B9 and Pf but there was an increased sensitivity to the f116 phage.

Examination of *P. aeruginosa* hosts containing the mutant pilin genes by electron microscopy revealed that they were all piliated although differences in the amount of piliation was obvious: wild-type pilin (~20 pili per cell), Gly-113-to-Glu pilin (~5 to 10 pili per cell), Pro-72-to-Leu pilin (~3 pili per cell), and Ser-131-to-Asn pilin (~1 pilus per cell). These differences in piliation did not appear to affect sensitivity to B9 or f116 markedly, indicating that the changes in phage susceptibility was attributed to altered receptor-binding domains as a result of the point mutations and not due to variable degress of piliation.

2. Bacteriophage Susceptibility of *P. aeruginosa* PAK and PAO1-leu.

The wild-type PAK and PAO1-leu strains were assayed against the four bacteriophage (Table 4). PAK was more sensitive to PO4, B9 and Pf than PAO1-leu and in fact, PAO1-leu was totally resistant to Pf as noted earlier by Bradley (4). Only f116 was more lytic in PAO1-leu than in PAK.

3. Bacteriophage Susceptibility of *P. aeruginosa* Expressing Each of the Five Different Pili Types.

The pilin genes from five different pilin prototypes (17) were cloned into pUC19 and then into the broad host range vector pKT210 as described in "Materials and Methods". Another pilin gene almost identical to the PAK pilin gene, from strain RMCK1, was also cloned in this manner. The six different pilin clones and the control vector, pBP090, were mobilized into BLP2 and BLP3 and assayed for their sensitivity to each of the four different bacteriophage using the double-agar-layer plate technique (Table 3).

Both BLP2 and BLP3 remained resistant to all four phage when harboring the control vector, pBP090. Bacteriophage B9 was able to infect all 12 strains harboring a pilin gene, indicating that all the strains assembled pili. All the pili, except K122-4 pili, conferred *P. aeruginosa* sensitivity to f116. The only pili conferring high Pf phage sensitivity to BLP2 and BLP3 were the PAK, RMCK1, and CD pili. PO4 was able to lyse PAK cells about 1,000-fold more effectively than the PAO1-leu cells (Table 4). Consistant with these results were the data in which the PAK and RMCK1 pilin clones conferred a 1000-fold more sensitivity to PO4 in both BLP2 and BLP3 than did the PAO pilin clone. The CD pili also conferred high sensitivity to PO4. Cells assembling P1 and K122-4 pili were relatively resistant to PO4 phage.

In several instances, the degree of phage sensitivity was dependent upon the host expressing the pili. The sensitivity to f116 was consistently higher in the PAO background host than in the PAK host. Also, weak sensitivity to PO4 was observed with P1 pili expressed by BLP2 but not by BLP3 whereas some weak sensitivity to Pf was detected for K122-4 pili expressed by BLP3 but not by BLP2. As well, it should be noted that RMCK1 and PAK pili expressed by BLP2 resulted in equal sensitivities to Pf whereas the RMCK1 pilin was 100-fold less sensitive to Pf than PAK pilin in BLP3.

D. DISCUSSION

The amino acids exposed on the surface of the pilus structure are the residues which generate the receptor-binding domain for each of the bacteriophage. Mutations in the pilin sequence which prevent the infection of a pilus-specific phage but allow the assembly of pili can be assumed to be amino acids which are at the pilus surface. Another less likely possibilty is that the phage bind to the mutated pili but the pili do not retract which is a necessary step for infection. The experiments in this chapter do not distinguish between these two possibilities. Three different mutants of PAK pilin were collected which altered the susceptibility to pilus-specific bacteriophage. The Gly-113-to-Glu substitution completely abolished PO4 sensitivity both in the pilin-lacking PAK and PAO1-leu hosts, and it slightly increased the sensitivity to Pf in BLP2. Since PO4 adheres to the lateral surface of the pilus (3), Gly-113 is probably exposed along the side of the pilus. Another possibility is that Gly-113 has an internal location in the pilus structure and the substitution by such a different amino acid (Glu) significantly altered the conformation of the surface residues which comprise the PO4 receptor. Gly-113 may also be shared in the Pf receptor-binding domain since the mutation at this position affected Pf sensitivity.

The Ser-131-to-Asn mutation was located between residues cysteine-129 and cysteine-142 at the C terminus. The cysteines are in the oxidized state, resulting in a C-terminal disulfide loop (22). In the pilin-lacking PAK host, this mutation conferred total resistance to the filamentous phage, Pf. Since Pf binds to the pilus tip (4), it is probable that this site is the location where the disulfide loop is exposed.

The Pro-72-to-Leu mutation in PAK pilin did not exhibit a very strong change in phage sensitivity, although it did affect the sensitivity of each phage in BLP2 by 10-fold. This amino acid might be recognized by all the phage or it may alter the structure of other nearby domains. Of particular interest was the 10-fold increase in sensitivity to f116 in BLP2, suggesting that Pro-72 has a lateral location on the pilus.

As demonstrated by ELISA, PAK pilin peptides containing residues 1 to 30, 70 to 81, 82 to 110 (24) and 129 to 142 (8, 24) were the most antigenic regions of PAK pili. The mutations at Pro-72 and Ser-131 are positioned within two of the peptides identified. As well, Gly-113 is just outside the border of the peptide constituted by residues 82 to 110. Therefore, the biological data supports the immunological experiments in designating Pro-72, Gly-113, and Ser-131 as surface residues of PAK pili.

The NMePhe pili from *P. aeruginosa* PAK and from two different strains of *Bacteroides nodosus* are capable of assembling into pili in *P. aeruginosa* DB2 (5; Chapter VII). The pilin subunits from these four strains have no more than 65% homology with any of the other pilin subunits. The construction of isogenic, pilin-lacking mutants of strains PAK and PAO1-leu led to an easy assay for the assembly of heterologous, cloned pilin genes by electron microscopy and by sensitivity to pilus-specific bacteriophage. We have shown that five different pilin types can be assembled in two different *P. aeruginosa* strains. Even the unusual characteristics of the P1 and K122-4 pilins did not prevent assembly. The pilins of *P. aeruginosa* strains P1 and K122-4 have weak homology to each other and to the PAK, PAO and CD pilins from positions 31 to 55. On the other hand, PAK, PAO and CD pilins are vitually identical in this area (17). K122-4 pilin has a 7 amino acid leader instead of a 6 amino acid leader peptide like the other 4 pilin types. P1 has a C-terminal disulfide loop of 17 residues compared to the 12 in the 4 other types (17). These results demonstrate the broad capacity of the assembly machinery of *P. aeruginosa* to accommodate highly heterologous pilins and that the most important structure of the pilin for assembly is the first 30 highly conserved, hydrophobic amino acids of the N terminus.

Each of the four bacteriophage had a different spectrum of pili which were used as receptors, indicating that each of the phage recognized a unique receptor-binding domain. The above data is supported by data from the point mutants. The Gly-113-to-Glu mutation completely abolished PO4 infectivity but did not appreciably affect susceptibility to the other three phage, indicating that this residue was important only to the PO4 receptor-binding domain. A similar point can be made for the Ser-131-to-Asn mutation and the Pf receptor-binding domain.

In several instances, the phage susceptibility profiles differed for a particular pili, depending on whether it was expressed in BLP2 or BLP3. One possibility for the observed differences between the two hosts is that the pilin was assembled differently in each host and resulted in structurally different pili and altered receptor-binding domains.

Bacteriophage B9 exhibited the broadest spectrum of specificity, infecting *P. aeruginosa* expressing any pili type, which reasons that a common receptor-binding domain existed for all five pili-types. The best candidate fulfilling this criteria is the highly conserved, N-terminus of the five pilins (17). Indeed, Watts *et al.* (24) identified the PAK peptide of residues 1 to 30 as an antigenic determinant and proposed that the first 9 amino acids may be responsible for the observed antigenicity. These residues might be exposed at the surface, available as a domain for B9 interaction.

Strain PA01-leu was totally resistant to the filamentous phage Pf. However, providing the CD or PAK pilin subunit to the pilin-lacking PA01-leu host conferred Pf sensitivity, thus providing evidence that PA01-leu has the proper machinery necessary for Pf replication but lacks the proper pilus receptor for Pf. Also, single point mutations at the C-terminus of the PAK pilin subunit (the arginine-144 to lysine substitution of RMCK1 pilin and the Ser-131-to-Asn pilin mutant) affected Pf infection, another indication that the pilin subunit itself constitutes the Pf receptor. Since Pf binds to the tips of PAK pili (4), and it is the pilin subunit which is the Pf receptor, we have concluded that *P. aeruginosa* pili do not have a specific tip protein. In the type 1 and type P pili systems of *E. coli*, the adhesins are minor components located at the pilus tip (6, 9). In contrast, it is the pilin subunit itself of K88 pili in *E. coli* which is the adhesin. A single amino acid substitution (Phe-150-to-Ser) within the pilin protein abolishes the adhesive property of these pili (7). More applicable to *Pseudomonas* pili, type-specific monoclonal antibodies to the disulfide loop of *N. gonorrhoeae* pili block adherence of the pili to buccal epithelial cells and they protect epithelial cells in a cytotoxic assay against *N. gonorrhoeae* (12, 23). These data imply that the disulfide loop of the pilin subunit is, at least, partly responsible for the adherence properties of N. *gonorrhoeae* pili. In similar studies, a monoclonal antibody to the disulfide loop of PAK pilin was able to limit adherence of *P. aeruginosa* PAK to buccal epithelial cells (P. Doig, P.A. Sastry, R.S. Hodges, K.K. Lee, W. Paranchych and R.T. Irvin, submitted). As well, a synthetic peptide with a sequence identical to residues 128 to 144 of PAK pilin is able to bind both buccal and ciliated trachael epithelial cells and it competitively inhibits the binding of purified PAK pili to buccal epithelial cells (R.T. Irvin, P. Doig, K.K. Lee, P.A. Sastry, W. Paranchych, T. Todd, and R.S. Hodges, submitted). Similarily, a proteolytically generated peptide from PAK pilin, encompassing residues 121 to 144, significantly inhibits the adherence of *P. aeruginosa* to buccal epithelial cells (13). Considering these data, we propose that it is the pilus tip which is responsible for adherence to epithelial tissue, and more specifically, the disulfide loop at the tip.

The phage sensitivity patterns obtained from *P. aeruginosa* assembling PAK, PAO and CD pili leads to some interesting speculation about pilus structure. The PAK and CD pili have almost identical phage sensitivity patterns which are different from the PAO profile, even though the primary structure of CD pilin is more homologous to the PAO (~80%) than to the PAK (~62%) pilin sequence (15). Seemingly, this data is contradictory unless it is hypothesized that critical amino acids, shared by both the PAK and CD pilins but different from the PAO pilin, generate similar domains at the CD and the PAK pilus surfaces, but produce a different domain at the PAO surface. One method of determining these critical surface residues is to align the three pilin protein sequences for the best homology and select the positions where the amino acids are identical between CD and PAK but differ with the PAO sequence (15). The amino acids selected from the PAK and CD sequences by this process were: Thr-84, Gly-113, Thr-130, Glu-135, and Ile-138. Most notably, Gly-113 also happens to be the residue which was mutated in PAK pilin to confer total resistance to PO4. Another amino acid selected by this process, Thr-130, is immediately adjacent to Ser-131 which was another residue mutated in PAK pilin to confer Pf resistance. The fact that these predictions are consistent with the biological and immunological data, provide compelling evidence that Gly-113 and Ser-131 are located at the pilus surface. Moreover, three of the five residues selected (Thr-130, Glu-135, and Ile-138) were within the disulfide loop, providing additional evidence that Pf recognizes the disulfide loop.

The original strains, from which the K122-4, RMCK1, and P1 pilin genes were cloned, were weakly piliated or bald. Thus, establishing the phage sensitivity patterns of these strains was impossible. Therefore, these newly constructed strains will be useful for identifying other bacteriophages which use P1 and K122-4 pili specifically as their receptors and this could lead to more information about pilus structure. Equally important, these strains will allow the binding constants of each of the pili prototypes to be assayed in the same background host and thus determine which of these prototypes adheres to epithelial tissue most effectively.

FIGURE IX.1

Construction of the clones containing the pilin genes from *P. aeruginosa* strains PAK, RMCK1, PAO, CD, K122-4, and P1. See "Materials and Methods" for construction details.

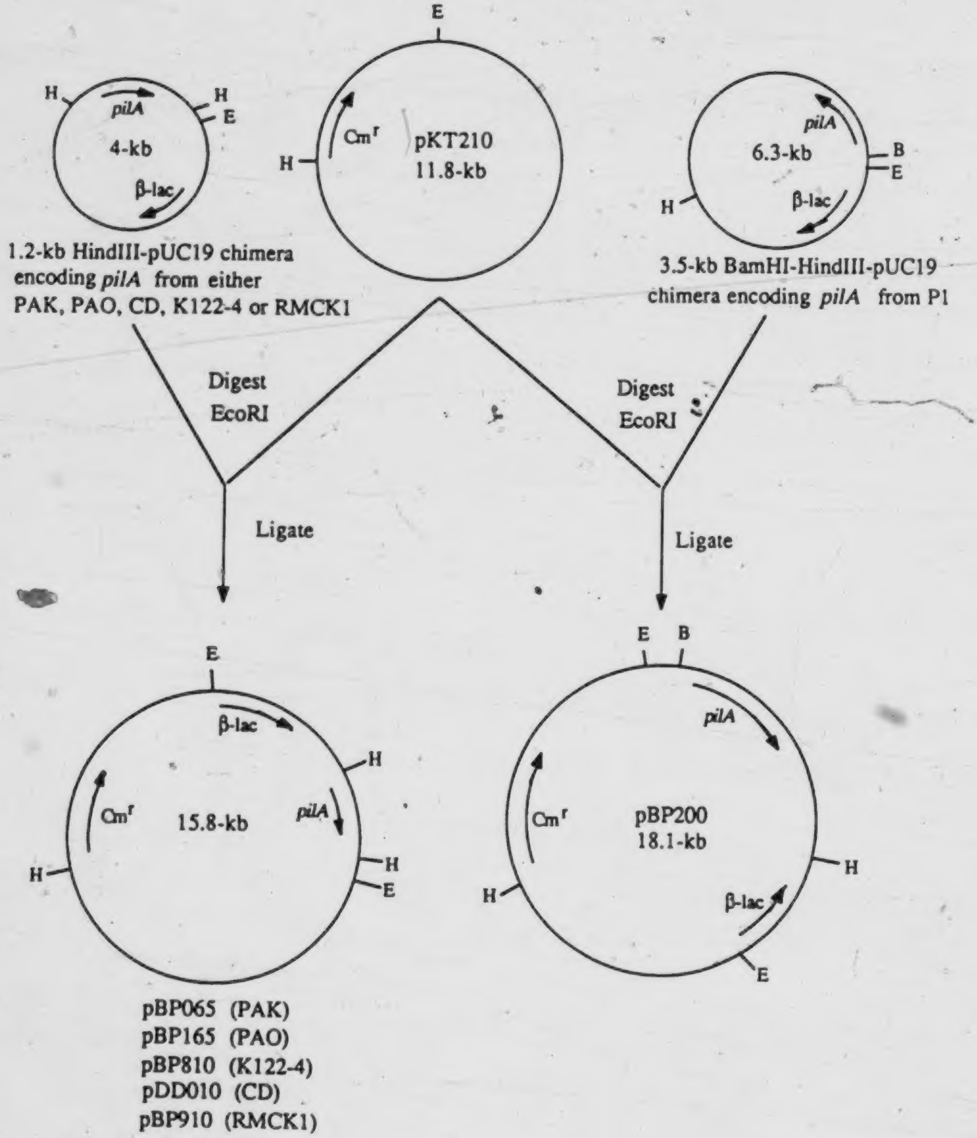
E
H
H
E
pilA
4-kb
β-lac
Cm^r
pKT210
11.8-kb
H
pilA
6.3-kb
B
E
H
β-lac
1.2-kb HindIII-pUC19 chimera encoding pilA from either PAK, PAO, CD, K122-4 or RMCK1
3.5-kb BamHI-HindIII-pUC19 chimera encoding pilA from P1
Digest EcoRI
Digest EcoRI
Ligate
Ligate
E
β-lac
H
Cm^r
15.8-kb
pilA
H
H
E
E
B
pilA
Cm^r
pBP200
18.1-kb
H
H
β-lac
E
pBP065 (PAK)
pBP165 (PAO)
pBP810 (K122-4)
pDD010 (CD)
pBP910 (RMCK1)

TABLE IX.1

Bacterial Strains, Plasmids and Bacteriophage.

Strain	Relevant phenotype	Reference or source
Bacteria		
Escherichia coli		
JM83	Δ(*lac-proAB*), *lacZ*ΔM15	25
MM294(pRK2013)	Helper plasmid for mobilization	20
DH5α	*hsdR17* ($r_K^- m_K^+$) *lacZ*	17
Pseudomonas aeruginosa		
PAO1-leu	*leu*$^-$ mutant of PAO1 (ATCC 25247)	W. Paranchych
PAK	Auxotroph	ATCC 25102
PAKp	Pilin minus mutant of PAK	16
BLP2	Tn*501*::*pilA* of PAK, Hg^r	Chapter VIII
BLP3	Tn*501*::*pilA* of PAO1-leu, Hg^r	This study
RMCK1	Cystic fibrosis isolate, PAK-like pilin	D. Speert[a]
Plasmids		
pUC19	MCS, Ap^r	25
M13mp18	Sequencing vector with MCS	25
M13mp19	Sequencing vector with MCS	25
pKT210	Broad host range cloning vector	1
pMR5	Temperature-sensitive, IncP plasmid, Cb^r	19
pBP311	Tn*501*::*pilA* of pBP310	Chapter VII
pBP500	PAK *pilA* clone, Ap^r, Cm^r	16
pBP502	Pro-72 to Leu mutant of pBP500	This study
pBP503	Gly-113 to Glu mutant of pBP500	This study
pBP504	Ser-131 to Asn mutant of pBP500	This study
pBP090	Chimera of pUC19 and pKT210	This study
pBP065	PAK *pilA* clone, Ap^r, Cm^r	This study
pBP165	PAO1-leu *pilA* clone, Ap^r, Cm^r	This study
pBP200	P1 *pilA* clone, Ap^r, Cm^r	This study
pBP810	K122-4 *pilA* clone, Ap^r, Cm^r	This study
pBP910	RMCK1 *pilA* clone, Ap^r, Cm^r	This study
pDD010	CD *pilA* clone, Ap^r, Cm^r	This study
Bacteriophage		
PO4	Noncontractile tail, side binding	3
f116	Noncontractile tail, side binding	18
Pf	Filamentous, tip binding	11
B9	Noncontractile tail, side binding	R. Hancock[b]

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b. R.E.W. Hancock, Dept. Microbiology, Univ. of British Colombia, Vancouver, British Colombia, Canada V6T 1W5.

TABLE IX.2

Mutations in PAK Pilin.

Clone	Position in pilin sequence	Wild-type amino acid (codon)	Mutant amino acid (codon)
pBP502	72	Pro (CCT)	Leu (CTT)
pBP503	113	Gly (GGG)	Glu (GAG)
pBP504	131	Ser (AGT)	Asn (AAT)

TABLE IX.3

Phage Sensitivities of the *P. aeruginosa* Strains PAK and PAO1-leu Pilin-Lacking Mutants Expressing the Five Pili Prototypes and the PAK Pilin Point Mutants.

Pili expressed (clone)	Lowest titre producing confluent lysis[a]							
	P. aeruginosa host							
	PAK Host (BLP2)				PAO Host (BLP3)			
	Bacteriophage							
	PO4	B9	Pf	f116	PO4	B9	Pf	f116
PAK (pBP500)	6	6	6	7	5	7	6	5
Leu-72 PAK (pBP502)	7	7	7	6	5	7	6	5
Asn-131 PAK (pBP504)	7	8	R	7	5	7	6	5
Glu-113 PAK (pBP503)	R	6	5	7	R	7	6	5
PAK (pBP065)	6	7	7	7	5	7	6	5
RMCK1 (pBP910)	6	7	7	7	5	7	8	7
PAO1-leu (pBP165)	9	8	R	7	8	7	R	5
CD (pDD010)	5	6	5	6	5	7	6	5
K122-4 (pBP810)	R	7	R	R	R	7	10	R
P1 (pBP200)	9	6	R	7	R	7	R	7
Vector (pBP090)	R	R	R	R	R	R	R	R

a. The numbers indicate the $\log_{10}$ of the phage titre. The R indicates complete resistance to the phage at the highest titre.

TABLE IX.4

The Phage Sensitivity Patterns of *P. aeruginosa* Strains PAK and PAO1-leu.

P. aeruginosa strain	Lowest titre producing confluent lysis[a]			
	Bacteriophage			
	PO4	B9	Pf	f116
PAK	4	6	5	7
PAO1-leu	7	7	R	5

a. The numbers indicate the $\log_{10}$ of the phage titre. The R indicates complete resistance to the phage at the highest titre.

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Chapter X

DISCUSSION

To begin a detailed molecular analysis of the NMePhe pilin of *Pseudomonas aeruginosa*, the pilin genes from several isolates were cloned and sequenced. A comparative study of the cloned pilin genes determined the course for the experiments which followed. The studies eventually focused upon three distinct regions of the gene: 1) the N terminus of the pilin protein and the effects within this area which affect pilin maturation and assembly, 2) the C-terminal region of the pilin subunit and its location on the pilus surface, and 3) a DNA sequence upstream of the pilin promoter (*sipR*) involved in the positive regulation of the pilin gene. The discussion below will briefly summarize the main results and conclusions of this thesis, compare them to other work in the field, and suggest directions in which future experiments should take.

A. PILIN POLYMORPHISM

The pilin genes from many isolates of *P. aeruginosa* were cloned and sequenced (Chapter III) to gain an understanding of the antigenic variation detected in the pili from different strains (Bradley and Pitt, 1975). In conjunction with these studies, it was demonstrated that the pilin genes were encoded as a single copy per cell, unlike *Neisseria gonorrhoeae* and *Moraxella bovis* which have multiple copies. A homology study of five different *P. aeruginosa* translated pilin gene sequences led to the observation that the mature pilin could be partitioned into four regions. The first 30 amino acids of the N terminus are highly conserved and strongly hydrophobic in all five pilin types. A semivariable region continues from the conserved sequence to residue 54. Located immediately after this domain, within the center of the pilin sequences, is the hypervariable domain which has been designated the immunodominant epitope of PAK pilin (Watts *et al.*, 1983). The last third of the pilin (which includes the disulfide loop) is a semiconserved domain.

Presently, it is not known whether the variation of pili serves any purpose in *P. aeruginosa*. Since there appear to be a limited number of pilin prototypes and the sequences of these pilin genes are quite stable over time (Pasloske *et al.*, 1988), it is not likely that they are a mechanism of evading the host immune system as they are in *N. gonorrhoeae* where pilin variants are easily isolated from the same infected host in a short span of time (Nicolson *et al.*, 1987). Instead, sequence changes are probably due to genetic drift or a recombination event with extracellular DNA from another NMePhe organism. The changes which survived were those mutations which were tolerated within the limits set by the requirements for perpetuating the pilus structure.

B. PILIN MATURATION AND ASSEMBLY

Results from DNA sequencing revealed that pilin was translated as a precursor form, having an amino-terminal extension of 6 or 7 amino acids which are positively charged (Chapters II and III). Immediately following the leader peptide is the hydrophobic N-terminal region. These two features are characteristic of the signal sequences used by proteins to cross biological membranes (Michaelis and Beckwith, 1982). The resemblance of the prepilin N-terminal structure to the signal sequences suggested that it had a similar function.

Studies in *E. coli* demonstrated that a peptide having the sequence of the first 36 residues of prepilin was capable of associating with the membrane fraction, implicating the importance of this sequence in pilin transport (Chapter V).

Expression experiments in *P. aeruginosa* demonstrated that deletions of 4 or 8 amino acids in the mature portion of the N terminus prevented leader peptide removal and pilus assembly (Chapters VI and VII). The glutamate at position 5 (Glu-5) substituted by a lysine (Lys) inhibited phenylalanine methylation but did not prevent leader peptide removal suggesting that Glu-5 is recognized by the transmethylase but not the leader peptidase. Glu-5 is strictly conserved among all the NMePhe pilins sequenced implying that it has some underlying importance. The relevance of pilin methylation is not known. The Glu-5-to-Lys pilins do not assemble into pili unless they polymerize in the presence of wild-type subunits. The dysfunctional polymerization of Glu-5-to-Lys pilin could be attributed to the unmethylated phenylalanine or to the Glu-5 substitution. N-terminal protein sequencing data obtained from the pili of *N. gonorrohoeae* and

N. meningitidis concluded that the assembled pilins had N-terminal heterogeneity (Hermodson *et al.*, 1978). Half of the pilin began with NMePhe and half had N-terminal threonines. In this case, lack of the NMePhe residue did not prevent pilus assembly suggesting that this residue is not required for assembly. Therefore, Glu-5 is probably important in subunit-subunit interactions in the assembled pilus and recognition by a methylase.

To delineate the substrate peptide sequences recognized by the leader peptidase and the transmethylase, construction of other intelligently chosen mutations within the N-terminal region of the prepilin are required. The novelty and the lack of literature concerning NMePhe pilin maturation, transport and assembly make this area of research deserving of much more experimentation.

C. THE CARBOXY TERMINUS

Chemical mutagenesis was used to produce three different point mutations within the pilin gene of *P. aeruginosa* strain PAK (Chapter IX). The mutant pilins were partly characterized by determining the susceptibilities of *P. aeruginosa*, expressing these pilins as pili, to pilus-specific phage.

Two of the three mutant pilins were very interesting. One of the mutant pilins had a substitution of the glycine at position 113 (Gly-113) by a glutamate which obliterated the infectivity of bacteriophage PO4. The receptor for this phage has a lateral location along the pilus and therefore, Gly-113 might be exposed along the length of the pilus.

In contrast, a substitution located within the disulfide loop, of a serine at position 131 (Ser-131) by an asparagine, resulted in *P. aeruginosa* becoming resistant to the tip-binding phage, Pf. This data indicated that the disulfide loop may be exposed at the pilus tip. Interestingly, the receptor-binding domain for human buccal and trachael epithelial tissue is, at least in part, constituted by the disulfide loop (P. Doig, P.A. Sastry, R.S. Hodges, K.K. Lee, W. Paranchych, and R.T. Irvin, submitted). If this is the case, there ought to be amino acids in common among the different pilin prototypes in the C-terminal disulfide loop.

An examination of all the known C-terminal, NMePhe pilin sequences demonstrates that the C-terminal sequences of various pilin types can be divided into into four distinct groups (Fig. 1). Group III consists of only *Bacteroides nodosus* pilins and it is unique in that these pilins do not have a C-terminal disulfide loop. However, these sequences are homologous with the other groups. Each of the three other groups have C-terminal disulfide loops and they are distinguished from each other by the number of amino acids within the loop and by the sequence homology that they have to each other within the loop.

Group I is comprised of pilins from *P. aeruginosa* and *M. bovis* which have 17 or 18 amino acids within the disulfide loop, respectively. Group II has pilins with 12 amino acids in the loop from *P. aeruginosa* and *B. nodosus* while group IV has pilins with approximately 30 amino acids in the loop from *N. gonorrhoeae* and *Neisseria meningitidis*.

The C termini of the four groups have been divided into domains A, B, C, and D. Domains A and C are conserved in sequence homology while domains B and D are variable. Within each of the groups, certain positions are conserved in domain B which are not homologous with the same position in other groups. One such example is the strongly conserved isoleucine in position 7 of the group I sequences which is not found in any other group.

The single position which is highly conserved in domain B of groups I, II, and III is that held by a serine or threonine at the fifth position of domain B. This was the same position which was mutated to confer resistance to the tip-binding phage, Pf.

Domain D is highly variable in sequence but it is characteristically charged. The predominant amino acid is lysine.

The conserved nature of domains A and C suggest they have functional relevance. Secondary structure predictions were not successful in elucidating a consistent structure among all the pilins in this region. Nonetheless, it is possible that either of the domains may be involved as the receptor-binding domain for the epithelial cell receptor or that the two domains may act synergistically. Notably, aromatic amino acids and the absolutely conserved proline (domain C) are the prominent residues in these two domains. Site-specific mutagenesis of these residues and their effects on the adherence of phage Pf and on the efficiency of binding to epithelial cells might demonstrate their importance as members of a receptor-binding domain.

Preliminary studies have demonstrated that peptides, encompassing the C-terminal sequence of *P. aeruginosa* PAK pilin, and antipeptide antibodies to the same peptide, block adherence of *P. aeruginosa* to human buccal and tracheal epithelial cells (unpublished data). Due to the profound nature of the above results, the efficacy of the C-terminal peptide as a vaccine will be tested in human volunteers in the near future. It is hoped that antibodies made to the peptide will block adherence of *P. aeruginosa* in the upper respiratory tract.

D. REGULATION

A region upstream of the pilin promoter was identified which enhanced pilin gene expression greater than 10-fold (Chapter VIII). Within this DNA fragment was a sequence which resembled a NifA-binding site ($TGTN_{10}ACA$), which is a regulatory sequence of the nitrogen fixation genes from *Klebsiella pneumoniae* (Buck *et al.*, 1986). It also had strong homology with the regulatory element II-1 from *Caulobacter crescentus*. The similarity to either of these regulatory sequences could be entirely fortuitous such that the recognition site within the regulatory fragment of the *P. aeruginosa* pilin gene (*sipR*) bears no resemblance to the recognition sequences of either of the previously mentioned elements.

Experiments as elegant as those performed by Morett *et al.* (1988) will be needed to delineate the nucleotide residues within *sipR* critical to pilin gene activation. These workers constructed many different site-specific mutations in the upstream activating sequence (NifA-binding site) and then assayed for transcriptional activation using a promoter probe vector.

The NifA-recognition sequence can be moved more than one kilobase upstream of the promoter and activity retained (Buck *et al.*, 1986), a characteristic feature of eukaryotic enhancer sequences. Can *sipR* be moved further away from the pilin promoter and maintain its enhancing properties? As well, can the orientation of the sequence be reversed?

Finally, it is important to identify and characterize the protein which activates pilin gene expression. Regulatory proteins of the NMePhe-like pilin gene from *Vibrio cholera* (ToxR; Miller *et al.*, 1987) and the NMePhe pilin gene of *N. gonorrhoeae* (PilA and PilB; Taha *et al.*, 1988) have been partially characterized. In *V. cholera*, pilin expression is dependent upon pH, salt concentration, and nitrogen availability as detected and mediated through ToxR (Peterson and Mekalanos, 1988). Presently, similar physiological studies are lacking for the expression of *P. aeruginosa* pili. The field of *P. aeruginosa* pilin gene regulation has many questions and it will be interesting to learn whether there will be any similarities to pilin gene regulation in *V. cholera* or *N. gonorrhoeae* or whether the regulatory systems will be as variable as the pilin genes they control.

FIGURE X.1

Alignment of the C-terminal sequences from NMePhe pilins. The sequences were aligned to obtain the best possible homology by visual examination on the basis of complete identity and conservative substitutions. The dashes are used for spaces to obtain the best possible alignment. The underlined regions in the group IV sequences indicate a variable region of approximately 15 amino acids. The periods in the consensus sequence indicate positions with a high degree of variability. Protein sequences were obtained from the following: *P. aeruginosa* TBOU1 and GA1 (unpublished data); *P. aeruginosa* PAK, PAO CD, P1, and K122-4 (Pasloske *et al.*, 1988); *B. nodosus* strains (Elleman, 1988); *M. bovis* strains (Marrs *et al.*, 1985); *N. gonorrhoeae* MS11 (Schoolnik *et al.*, 1984) and P9 (Nicolson *et al.*, 1987); *Neisseria meningitidis* C311 (Potts and Saunders, 1988). Abbreviations: B.n., *B. nodosus*; M.b., *M. bovis*; N.g., *N. gonorrhoeae*; N.m., *N. meningitidis*; P.a., *P. aeruginosa*.

BACTERIAL STRAIN	C-TERMINAL SEQUENCE	GROUP
P.a. P1	GVWNCKITKT-PTAWKPNYAPNACPKS	I
P.a. TBOU1	GVWGCSISST-PANWKPNYAPSNCPKS	
P.a. GA1	GVWTCGITGS-PTNWKTNYAPANCPKS	
M.b. alpha	GVWTCKIDGSQAAKYKEKFNPTGCVKKSK	
M.b. beta	GVWTCTINGSAAPGWKSKFVPTGCKE	
P.a. PAK	GLWKCT---SDQD---EQFIPKGCSK	II
P.a. PAO	GVWACK---STQD---PMFTPKGCDN	
P.a. CD	GVWTCT---STQE---EMFIPKGCNKP	
P.a. K122-4	GTWACT---SNAD---NKYLPKTCQTATTTTP	
B.n. D	GTWTCA---TTVE---AKFQPTGCKDGK	
B.n. H	ATWSCS---TDVD---EKFKPTGCKK	
B.n. A	GSYKYNEGETDLE---LKFIPNAVKN	III
B.n. B	GSFIAGDG-TDLA---DKFIPNAVKAKK	
B.n. C	GSYVKESA--TVK---DKFLPKALKETK	
B.n. E	GSYTQGDA--TLD---AKFIPNAVKKSQ	
B.n. F	GSYTQGGG--DID---PKFVPNAVKKVQ	
B.n. G	GSYDKDSS-STKV---PKFLPKALKEATP	
N.g. MS11	VKWFCGNPVTR______TKHLPSTCRDKASDAK	IV
N.g. P9	VKWFCGRPVKR______TKHLPSTCRDNFDAS	
N.m. C311	VKWFCGQPVTR______TKHLPSTCRDASDAS	
CONSENSUS SEQUENCE	GVWTC....S......PKFIPKGCKK SY T YL NAV	
DOMAINS	A B C D	

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APPENDIX

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